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# The Adaptive Period for Foreign Antigens in Ontogenesis in Ducks

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*Received January 11, 1957*

In previous work, specific suppression on the formation of heteroagglutinins was found following long-term, repeated postembryonic injections of foreign blood in ducks (Hašková and Pokorná 1956). The most important factor in the origin of this non-reactivity of ducks to hen erythrocytes is adaption of the organism in the immediate postnatal period. A further analysis of the origin of this non-reactivity led to findings on the character, duration and significance of this period (i. e. the adaptive period), when the administration of foreign blood results in inhibition of the formation of heteroagglutinins in fully grown birds.

## *Methods*

Peking ducks, which had hatched out on the same day and had been reared under the same conditions, were used for the experiments. These were given 1—15 intravenous injections of fresh hen or goose blood (one part 3.8% citrate to nine parts blood) on alternate days, in amounts of 0.3—1 ml., according to the scheme given in the tables.

*Immunisation* was carried out in the experimental and control birds at exactly eight weeks, with four doses of 1.5 ml. blood on alternate days; blood was collected on the fifth day after immunisation had been completed. Re-immunisation was carried out at 13 weeks, with four doses of 1.5 ml. hen blood or 4 ml. goose blood, and blood was again collected five days after completion of the immunisation series. The control ducks received their first injections at eight weeks.

In order to determine normal reactivity of ducks to a foreign erythrocyte antigen, some of the ducks were immunised with four doses of 1.5 ml. citrated turkey blood; blood was again collected on the fifth day after completion of the immunisation series.

*Agglutination* was carried out by taking two drops of serum which had been stored for 24 hours at −20° C and one small drop of a 50% suspension of erythrocytes washed three times in physiological saline and incubating at room temperature. The results were read off after 10 and 60 minutes. The titres given in the tables are the inverted values of the final dilution of the serum which gave agglutination visible to the naked eye after 60 minutes.

*Incomplete antibodies* were determined by agglutination in protein medium and by the Coombs test (Dunsford and Bowley 1955). Antiglobulin serum was prepared according to the method suggested by Milgrom et al. (1956), by immunising a cockerel with its own erythrocytes, agglutinated by immune duck serum.

*Skin grafting:* When applying homografts, the skin was always exchanged from the middle of the back, between ducks of the same age. After turning the skin by 180°, the graft was sutured by eight stitches and lightly covered with collodion. Evalution of a permanent take was based on growth of the feathers in the reverse direction. In heterografts in adult ducks, a piece of skin measuring approximately 2 × 2 cm. was taken from the leg of a goose, sutured into position and lightly covered with collodion.

## *Results and Discussion*

It was found that long-term, repeated injections of hen blood in ducks, if commenced within six days of hatching, lead to complete inhibition of the formation of heteroagglutinins in fully grown birds. If the series of injections is commenced

Table 1. The Influence of a Series of Postembryonic Injections of Hen Blood Commenced on Various Days after Hatching, on the Formation of Heteroagglutinins in Ducks.

| Day after hatching on which injections commenced | No. of injections | Total amount injected (ml.) | Titre | | Titre of incomplete antibodies |
|---|---|---|---|---|---|
| | | | after first immunisation series | after second immunisation series | |
| 3 | 15 | 6.3 | 1 | 2 | 0 |
| 3 | 15 | 6.3 | 2 | 1 | 0 |
| 6 | 15 | 6.6 | 2 | 2 | 0 |
| 6 | 15 | 6.6 | 0 | 1 | 0 |
| 8 | 15 | 6.9 | 8 | 2 | |
| 8 | 15 | 6.9 | 16 | 16 | |
| 15 | 15 | 7.5 | 2 | 4 | |
| 15 | 15 | 7.5 | 4 | 8 | |
| 18 | 15 | 8.1 | 1 | 2 | |
| 18 | 15 | 8.1 | 256 | 64 | |
| 23 | 15 | 8.7 | 64 | 16 | |
| 23 | 15 | 8.7 | 32 | 16 | |
| — | — | — | 64 | 32 | |
| — | — | — | 16 | 32 | |
| — | — | — | 32 | 32 | |
| — | — | — | 64 | 32 | |

between the 8th and the 18th day, some ducks form immune heteroagglutinins to the same titre as the controls, while others do not form them to a titre higher than that for natural heteroagglutinins (Hašková and Pokorná 1956). If the series is commenced on the 23rd day, the ducks react in the same way as the control birds not given injections before immunisation (tab. 1, fig. 1). Since the period when the first injections are administered is the decisive factor, the authors are of the opinion that formation of immunological tolerance similar to that resulting from embryonal parabiosis or intraembryonal injections (Hašek 1953, Billingham, Brent and Medawar 1953) is involved. It confirmed the conclusion of the preceding communication (Hašková and Pokorná 1956).

For postembryonal injections in ducks, goose blood was also used, and it was found that the same inhibition of formation of heteroagglutinins occurred in adult life following a series of 15 postembryonal injections in doses of 0.3—0.7 ml. on alternate days, five injections of 0.3 ml. or two injections of 0.3 ml. on alternate days, or even after a single injection of 1 ml. goose blood, i. e. if a sufficient amount of antigen was administered up to the 10th—13th day after hatching (tab. 2, fig. 2). In non-reacting ducks, the presence of incomplete antibodies was not demonstrated either by agglutination in protein medium, or by the Coombs test. From all the above results it is concluded that the period in which ducks are capable of adaptation to a foreign antigen (i. e. the adaptive period) extends for several days into post-embryogenesis. The length of the adaptive period depends on the nature of the antigen. For goose blood it lasts, in all ducks, up to about 10—13 days and for hen blood cells up to six days after hatching. This period varies considerably however, among individual birds. Some ducks are capable of adaptation to hen or goose blood as late as 18 days after hatching; others do not develop tolerance when injected with antigen on the 8th day after hatching.

The origin of non-reactivity depends, without any doubt, on the dose of antigen administered during the adaptive period. When a single dose of 0.3 ml. goose blood

was injected during the adaptive period, it had no effect on the formation of antibodies at eight weeks. When the same amount was split up into three doses and administered on the first, third and fifth days, or when a single dose of 1 ml. was administered, the same inhibition of antibody formation was found as following

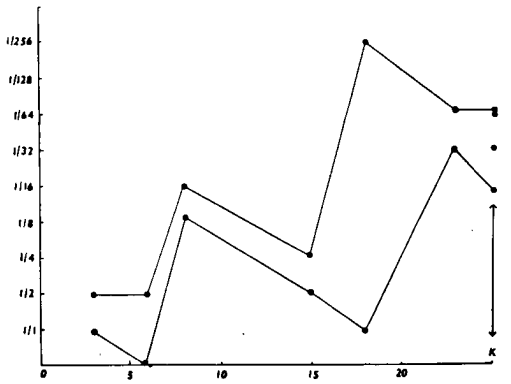


Fig. 1.

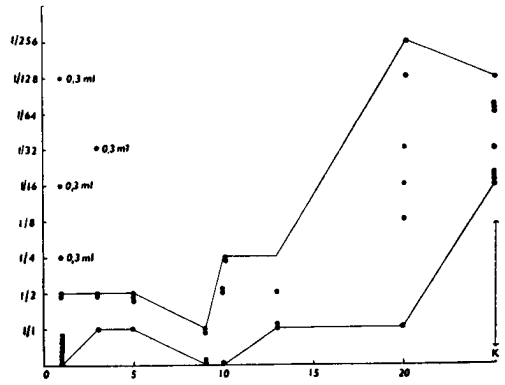


Fig. 2.

Fig. 1. Titres of heteroagglutinins against hen erythrocytes after immunisation at the age of eight weeks in ducks that had been given a series of 15 injections of hen blood, starting on varying days after hatching. $x$: day after hatching when the ducks received the first injection. K denotes the titres in the control ducks. $y$: last dilution of serum which gave visible agglutination.

Fig. 2. Titres of heteroagglutinins against goose erythrocytes after immunisation at the age of eight weeks in ducks which had been given one or several injections of goose blood starting on varying days after hatching. $x$: day after hatching on which ducks received first injection .K denotes titres of control ducks. $y$: last dilution of serum which gave visible agglutination. The mark "0.3 ml." denotes that the ducks indicated were given only the border-line amount of 0.3 ml. in the adaptive stage and that therefore their titres lie within the limits of the titres of the controls.

a series of injections (tab. 2). From this it is concluded that the important factor is not so much the amount of antigen administered in a given dose, but that the antigen should remain in the body for a certain time during the adaptive period, in order to be able to influence the mechanism of antibody formation.

Strong suppression of the formation of antibodies, against soluble heterologous proteins, however, was successfully obtained by Hanan and Oyama (1954) and by Dixon and Maurer (1955), following a series of postembryonal injections. It is evident that in these experiments also the organism was adapted to the antigen primarily in the adaptive period, shortly after birth. In the experiments described in the present communication, the formation of heteroagglutinins was completely inhibited; this had previously been successful only after turkey-hen parabiosis (Hraba 1956). Intraembryonal injections of foreign blood resulted at most in a decrease in the formation of heteroagglutinins, but never in complete inhibition (Hašek and Hraba 1955, Simonsen 1955, 1956).

Homografts were carried out in ducks on various days after hatching out, in order to verify the duration of the adaptive period. In five-day-old ducks, the growth of feathers in homografts was 100%, in seven-day-old birds 50%. This confirms that the adaptive period in ducks extends into postembryonic life for several days. Hraba and Hašek (1956) also obtained complete growth of feathers in homografts on one-day-old ducks. The lower percentage of their successful homografts is probably due to the different technique used (tab. 3).

Table 2. The Influence of Injections of Goose Blood on the Formation of Heteroagglutinins in Ducks.

| Day when first injection administered | Dosage scheme (injections given on alternate days) | No. of birds | Titres | |
|---|---|---|---|---|
| | | | after first immunisation series | after second immunisation series |
| 1st | 0.3 ml. | 2 | 128, 4 | 256, 32 |
| | 1 ml. | 2 | 0, 2 | |
| | 0.1 + 0.1 + 0.1 | 3 | 0, 0, 16 | |
| | 0.3 + 0.3 | 2 | 0, 0 | 0, 0 |
| | 0.3 + 0.3 + 0.3 + 0.3 + 0.3 | 2 | 2, 0 | 64, 0 |
| | 0.3 to 0.6 in 15 injs. | 2 | 0, 0 | 0, 0 |
| 3rd | 0.3 | 2 | 32, 2 | 128, 16 |
| | 0.3 + 0.3 | 1 | 2 | 0 |
| | 0.3 + 0.3 + 0.3 + 0.3 | 1 | 1 | 64 |
| 5th | 0.3 to 0.6 in 15 injs. | 1 | 2 | 2 |
| | 0.3 + 0.3 | 2 | 2, 2 | 1, 2 |
| | 0.3 + 0.3 + 0.3 + 0.3 + 0.4 | 1 | 1 | 8 |
| 9th | 1.0 | 2 | 1, 1 | |
| | 0.3 + 0.4 + 0.4 | 2 | 0, 0 | |
| 10th | 0.3 + 0.3 | 2 | 4, 2, 0 | 32 |
| | 0.3 + 0.4 + 0.4 + 0.4 + 0.4 | 2 | 2, 4 | 32 |
| 13th | 0.4 + 0.4 | 2 | 4, 1 | |
| | 0.4 + 0.4 + 0.4 + 0.5 + 0.5 | 2 | 1, 2 | |
| 20th | 0.4 + 0.5 | 2 | 16, 8 | |
| | 0.4 + 0.5 + 0.5 + 0.5 + 0.5 | 3 | 1, 32, 256 | |
| | 0.4 to 0.7 in 15 injs. | 1 | 128 | |
| Control birds of same age, injected at age of eight weeks | | 9 | 16, 128, 16, 32, 64, 64, 16, 64, 16 | 64, 512, 128, 256 |

Total number of birds used for the experiment: 48.

Table 3. The Proportion of Feathered Homografts and Others Carried out in Ducks on Various Days after Hatching

| Days after hatching | | | | | |
|---|---|---|---|---|---|
| 1 | 5 | 7 | 10 | 16 | 20 |
| 11/18 (Hraba and Hašek 1956) | 5/5 | 2/4 | 0/6 | 0/4 | 0/4 |

Skin grafts from the legs of geese were also made in ten-week-old ducks in which the formation of heteroagglutinins had been inhibited. These heterografts did not, however, survive longer than 15 days, either in ducks which did not form agglutinins on immunisation, or in the controls.

It is known that an injection of blood from another bird of the same species, when administered during the adaptive period, can result in permanent survival of a skin homograft, as the leucocytes and the skin have the same antigens in common. Injections of blood from a different species, although causing complete inhibition of the formation of heteroagglutinins did not, in these experiments, lead to prolonged survival of heterografts in adult birds in a single case. The question of heterografts is discussed elsewhere (Hašková and Hašek 1957).

*Summary*

1. Complete inhibition of the formation of heteroagglutinins in adult life was obtained in ducks by means of postembryonal injections of goose or hen blood. The experiments constituted a further demonstration of immunological tolerance.
2. It was found that the adaptive period for foreign erythrocytic antigens in ducks extended for 6—13 days into postembryogenesis.
3. The duration of the adaptive period depends not only on the species of the experimental birds, but also on the nature and degree of heterogenesity of the antigen, and even displays individual variations.
4. The important factor in the development of adaptation to a foreign antigen is not only the amount of antigen administered during the adaptive period, but rather the length of time over which it can act during this period. A single dose of 0.3 ml. administered during the adaptive stage did not affect antibody formation, but the same amount administered in three separate doses resulted in inhibition of the formation of heteroagglutinins in maturity.
5. Inhibition of the formation of immune heteroagglutinins did not lead to the development of incomplete antibodies.
6. The administration of blood during the adaptive period did not result in prolongation of the survival of heterografts.
7. Homografts carried out in ducks from 5—7 days after hatching out took permanently.

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# Адаптивный по отношению к чужеродным антигенам этап в онтогенезе уток

В. ГАШКОВА

*Резюме*

Мы установили, что с помощью повторных впрыскиваний куриной крови можно у взрослых уток полностью подавить образование гетероагглютининов после иммунизации при условии, что уколы были начаты не позже 6-го дня после выклевывания (срв. также Гашкова, Покорная 1956). Если серия уколов была начата на 8—18-ый день, у некоторых уток иммунные агглютинины образуются в таком же титре, как и в контроле, а у других — в титре не выше титра естественных гетероагглютининов. Если серия уколов была начата на 23-ий день после выклевывания, подопытные утки реагируют так же, как и контрольные, без уколов перед иммунизацией (табл. 1, график 1). При этих опытах дело идет опять об иммунологической толеранции (как в результате эмбрионального парабиоза или внутризародышевых впрыскиваний — Гашек 1953, Billingham, Brent и Medawar 1953). Для впрыскиваний после выклевывания мы использовали также гусиную кровь и убедились, что такого же подавления образования гетероагглютининов во взрослом состоянии, как после серии 15 постэмбриональных впрыскиваний от 0,3 до 0,7 мл через день, можно добиться с помощью 2 уколов по 0,3 мл через день или даже одноразового впрыскивания 1 мл гусиной крови, т. е. при условии, что достаточное количество антигена было введено не позже 10—13-го дня после выклевывания (табл. 2, рис. 2). Период, когда утки еще способны адаптироваться к чужеродным эритроцитарным антигенам (адаптивный этап) затягивается до 6—13-го дня постэмбриогенеза. Длительность адаптивного этапа зависит, повидимому, не только от вида животного, но и от характера и степени чужеродности антигена, а также проявляет и индивидуальные колебания. Дальнейшие опыты показали, что для возникновения адаптации к чужеродному антигену нет необходимости во введении антигена в течение адаптивного этапа только в каком-нибудь определенном количестве, а скорее — в достаточно долгом действии антигена в течение адаптивного этапа. Одноразовое введение 0,3 мл в течение адаптивного периода не оказывало влияния на образование антител, введение же этого количества тремя дозами по 0,1 мл вызвало подавление образования гетероагглютининов во взрослом состоянии. Наличие неполных антител не было нами доказано ни в одном случае. Продлить время выживания гетеротрансплантатов путем введения крови донора в течение адаптивного периода не удавалось. Гомотрансплантаты, пересаживаемые уткам до 5—7-го дня после выклевывания, устойчиво приживают.

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# Immunological Tolerance to Non-cellular Antigens

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Not long after it had been found that tolerance to foreign cell antigens could be produced (Hašek 1953, Billingham, Brent and Medawar 1953, Ripley, cit. by Owen 1954), it was demonstrated that a similar state could be induced using heterogenous proteins. In adult rabbits which had been injected during embryogenesis and the immediate postnatal period with equine serum albumin, Hanan and Oyama found absence of immunological response to this antigen. In rabbits which had received from birth large, repeated doses of human plasma or equine serum albumin, Dixon and Maurer (1955) found complete inhibition of the formation of precipitins against the antigen concerned. By injecting newborn rabbits with human seralbumin, Cinader and Dubert (1955) obtained complete suppression of antibody formation in later life. In hens which had been embryonal parabionts with turkeys, Hraba (1956) found depression, and in one case complete inhibition, of the formation of precipitins against turkey serum proteins.

The aim of the present work was to ascertain whether immunological tolerance to guinea-fowl serum proteins could be produced in chicks. The antigen was injected intra-embryonally, but because only small amounts could be administered in this way, larger doses were injected immediately after the chicks hatched out.

## *Methods*

Injections were administered into the yolk sacs of 4 to 5-day-old embryos by drilling a small opening in the shell in approximately the equatorial plane. The egg was illuminated and placed in such a way that the extra-embryonic vascular field was situated below the opening. The antigen was injected below the area vasculosa to a depth of about 0.5 cm. (from the shell) in the direction of the embryo. After completing the injection, the opening was closed with paraffin wax and the egg returned to the incubator. About 15—20% of these embryos hatched out, the most frequent cause of death being rupture of the yolk sac. Injections into the allanto-chorionic blood vessels of the embryo were carried out according to a method described in previous communications (Hraba et al. 1956, Hašek 1956).

The experimental birds were immunized and blood collected by the intravenous route. Guinea-fowl citrated plasma (1 part 3.8% sodium citrate to 9 parts blood) was used for all the injections and immunization doses, and guinea-fowl and hen plasma were used for the reactions. Precipitins were demonstrated by the ring reaction in miniature test-tubes; the titre is given according to the highest dilution of the antigen which still produced a discernible positive reaction. The antiserum was not diluted for the reactions and unless stated otherwise, the antigen was diluted in the geometric series: 10×, 20×, 40×, etc., commencing with tenfold dilution.

## *Results*

Guinea-fowl plasma was injected either intra-embryonally or into newly hatched chicks. Intra-embryonal injections were administered to three groups of chick embryos of different ages. For these injections a constant dose of 0.2 ml. was used.

Formation of Precipitins against Guinea-fowl Plasma in Experimental and Control Birds

| Titres in blood collected on 7th day after immunization (in group 4 on 7th day after second immunization dose). Antigen diluted in geometric series (1 : 10, 1 : 20, 1 : 40, etc.), commencing with tenfold dilution | | | | | |
|---|---|---|---|---|---|
| Groups of birds | Method of administration | Amt. of antigen | No. of birds | Titre of antibodies 7 days after administration | Diff. between exp. birds and controls eval. by t-test |
| 1. Exper. | Inj. in yolk sac in 4—5-day-old embryos chick | 0.2 ml. | 4 | 160; 320; 640; 1280 | t = 0.45 |
| Controls | — | — | 6 | 160; 160; 160; 320; 1280; 1280 | P = 0.70 |
| 2. Exper. | Inj. in allanto-chorionic blood vessels, 11—13-day-old chick embryos | 0.2 ml. | 12 | 70; 100; 100; 100;** 100; 100; 100; 100; 400; 400 | t = 0.68 |
| Controls | — | — | 10 | 100; 100; 100; 100; 100; 100; 400; 400; 400; 400 | P = 0.50 |
| 3. Exper. | Inj. in allanto-chorionic blood vessels in 18-day-old chick embryos | 0.2 ml. | 5 | 80; 160; 640; 640; 1280 | t = 0.5 |
| Controls | — | — | 7 | 160; 160; 160; 160; 640; 640; 640 | 0.7 > P < 0.5 |
| 4. Exper. | I. p. inj. 1st day after hatching | 1.0 ml. | 8 | 160; 160; 320; 1280; 1280; 1280; 1280; 5120 | t = 1.01 P = 0.30 |
| Exper. | I. p. inj. 1st and 2nd day after hatching | 3.0 ml. (1 + 1 + 1) | 9 | 40; 80; 80; 160; 160; 320; 1280; 1280; 5120 | t = 2.74 0.01 > P < 0.02 |
| Controls | — | — | 9 | 160; 320; 1280; 1280; 1280; 1280; 2560; 10240 | |

** Antigen diluted 1 : 10, 40, 70, 100, 400, 700, 1,000.

In the first group, guinea-fowl plasma was injected into the yolk sac of 4—5-day-old embryos. Transmission of these foreign proteins into the embryonic blood stream was demonstrated in embryos killed five days after the intravitelline injection had been carried out. In the other two groups, guinea-fowl plasma was injected into the allantochorionic blood vessel on the 11th—13th day and on the 18th day of incubation. All three groups of chicks which were given intra-embryonal injections of guinea-fowl plasma were immunized at the age of six weeks with a single injection of 1 ml. of the same antigen. All these birds formed antibodies and in no group was there any significant difference in the titre of the precipitins formed as compared with the controls.

Newly hatched chicks were injected intraperitoneally with guinea-fowl plasma. In the first group a single injection of 1 ml. was administered within 12 hours after hatching. In the second group three doses of 1 ml. were administered, within 12,

24 and 48 hours respectively after hatching. These birds were immunized with two injections of 1 ml. guinea-fowl plasma administered at the age of six and eight weeks after hatching. All birds again formed antibodies, but those which had received 3 ml. guinea-fowl plasma after hatching, formed precipitins against the antigen in a statistically significantly lower titre than the controls ($P < 0.02$) while in the group which had received only 1 ml. guinea-fowl plasma the titres did not differ significantly from those of the control birds.

*Discussion*

Hašek (1955) found that the injection of homologous blood led to the formation of immunological tolerance in about 50% of newly hatched chicks to a skin graft from the donor. These results indicate that the adaptive period, i. e. the period in which exposure to a foreign antigen leads to the development of immunological tolerance, does not end in chicks until after they hatch out, at least as far as homologous cells are concerned. It would appear, however, that the adaptive period for different antigens may end at different periods. Hašková (1957), for example, succeeded in producing tolerance to homologous cells in ducks up to the tenth day after hatching, whereas to hen erythrocytes the latest limit was the fifth day. It was not certain, therefore, whether it would be possible to produce immunological tolerance in chicks to guinea-fowl plasma after hatching out.

The experiments demonstrated that it is possible, but that relatively large amounts of antigen must be administered. This offers the most probable explanation for failure to produce immunological tolerance by means of intra-embryonal injections, in which only a small amount of antigen can be administered. The importance of the amount administered is evident from a comparison of the results in the two groups injected with guinea-fowl plasma after hatching out; a dose of 3 ml. led to the development of tolerance, while a dose of 1 ml. was ineffective. A relationship between immunological tolerance to turkey serum proteins and the amount of antigen injected into the chick embryo is also seen from the results obtained in chicks which were given intra-embryonal injections of whole turkey blood (Hraba et al. 1956); while tolerance to the serum proteins of the partner's species was found in hens which had been embryonal parabionts with turkeys, no reduction in the formation of precipitins against turkey serum was found in these other birds. With reference to the different methods of administering the antigen, it would, however, appear that apart from differences in the amount of foreign protein administered, other factors might also participate. For instance, Hašková (1957) found that in the case of postembryonal injection of hen blood in ducks, fractionation of the same dose at given intervals is more effective for inducing tolerance than administration of the whole amount in a single dose; for the induction of tolerance, therefore, it appears necessary for the antigen to act at least over a minimum given period.

Simonsen (1956) found that on the 17th—19th day of incubation of the chick embryo, immunological tolerance could be induced by a dose of human blood cells which at other periods would have no, or little effect.

Although injections of guinea-fowl plasma were administered from the 4th—18th day of incubation, no developmental period of increased sensitivity was found, in which a relatively small amount of this antigen could produce tolerance. It is assumed that these experiments do not demonstrate that no such sensitive stage to guinea-fowl plasma exists, as the negative results were plainly due to the fact that only sub-threshold amounts of antigen could be administered.

It will, however, be necessary to verify Simonsen's results apart from this, as when

chick embryos were injected with turkey blood from the 12th day of incubation up to the first day after hatching out, no reduction in the formation of agglutinins against turkey erythrocytes was found in any of the groups, although the same dose of foreign blood was used for injecting the embryos as had been used in Simonsen's experiments (Hraba et al. 1956).

On the other hand, the results in the group of newborn chicks and in chicks injected with guinea-fowl plasma administered into the yolk during early embryogenesis, together with the results of embryonal parabiosis between turkey and hen, are further evidence that the weak forms of tolerance induced in duck and hen embryonal parabionts are not due to termination of the adaptive stage for the partner species antigens before the time when the embryos are joined, i. e. the 11th—12th day of incubation, as assumed by Billingham et al. (1956).

The degree of immunological tolerance to guinea-fowl serum proteins obtained in these experiments, and the degree of tolerance to turkey serum proteins obtained in hen embryonal parabionts with turkeys, are lower than the degree of tolerance to foreign proteins induced in rabbits. This is probably due to differences in ability to acquire immunological tolerance in different species.

This relationship is particularly clear in duck-hen embryonal parabionts. On immunization at the same age, the duck parabionts form agglutinins against hen erythrocytes in lower titres than the controls, whereas in the hen parabionts the titres of agglutinins against duck erythrocytes are the same as in the controls (Hašek and Hraba 1955). A similar relationship can be seen in hens which received intraembryonal injections of turkey blood. Whereas parabiosis with this species produces marked or even complete suppression of the formation of agglutinins against turkey erythrocytes, the titres in the birds which only received injections of turkey blood did not differ from those in the controls (Hraba et al. 1956). On the other hand, in ducks injected with the same amount of goose blood as in the previous experiment, a reduction in the titre of antibodies against goose erythrocytes was found (Hašek 1956). In view of the fact that the acquisition of immunological tolerance to foreign proteins depends on the amount of antigen administered, it might naturally be possible that the difference between the reaction of hens and rabbits could be due to differences in the amount of protein injected. That this is not the case is demonstrated by the finding that complete inhibition of the formation of precipitins against the corresponding antigen was obtained in young rabbits by the injection of 20 mg. human or equine seralbumin (Cinader 1955, Smith and Bridges 1956). These are considerably smaller amounts of antigen than those required to produce only partial depression of the formation of precipitins against guinea-fowl plasma in chicks, showing that the rabbit belongs to the species in which immunological tolerance is acquired more easily than in hens.

*Summary*

Chick embryos were injected with amounts of 0.2 ml. guinea-fowl plasma, 1. on the 4th—5th day of incubation into the yolk sac, 2. on the 11th—13th and on the 18th day of incubation, into the allantochorionic vein. No decrease in the formation of precipitins was found in either of these groups following immunization with guinea-fowl plasma as compared with the controls.

Newly-hatched chicks were injected intraperitoneally with 1 ml. or 3 ml. guinea-fowl plasma. Following immunization, the chicks which had received 3 ml. guinea-fowl plasma formed precipitins against this antigen in a significantly lower titre than the controls ($P < 0.02$), while there was no significant difference between the titres in the birds which had received only 1 ml. and those in the controls.

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## Иммунологическое сближение по отношению к неклеточным антигенам

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*Резюме*

Вскоре после открытия иммунологического сближения по отношению к чужеродным клеточным антигенам (Гашек 1953, Billingham, Brent и Medawar 1953, Ripley 1953, цит. по Owen-у 1954) выяснилось, что подобного состояния можно добиться и по отношению к чужеродным белкам (Hanan и Oyama 1954, Dixon и Maurer 1955, Cinander и Dubert 1955 — у кроликов, Граба 1956 — у кур).

Целью настоящей работы было установить, можно ли добиться у цыплят иммунологического сближения с цесарками путем внутризародышевых и постэмбриональных впрыскиваний их сывороточных белков. Мы впрыскивали зародышам по 0,2 мл плазмы цесарки: 1) на 4—5-ый день инкубации в желточный мешок (переход чужеродных белков в кровяное русло зародыша был нами доказан); 2) на 11—12-ый или 18-ый день инкубации — в хорионаллантоидную

вену. После иммунизации 1 мл плазмы цесарки на 6-ой неделе после выклевывания ни в одной из этих групп не наблюдалось понижения способности к образованию преципитинов в сравнении с контролем. Немедленно после выклевывания цыплятам вводилось в полость брюшины по 1 или по 3 мл плазмы цесарки. После иммунизации цыплята, получившие 3 мл антигена, образовали преципитины в значительно более низком титре, чем в контроле ($t = 2{,}74$, $P < 0{,}02$). Титры цыплят, которым после выклевывания было введено только по 1 мл антигена, не отличались сколько-нибудь значительно от контроля.

Наши опыты показали, что иммунологического сближения у цыплят можно добиться и с помощью постэмбриональных впрыскиваний сывороточных белков цесарки. Но для достижения этого эффекта необходимо ввести сравнительно большое количество антигена, чем, повидимому, объясняется неуспех попыток вызвать его путем внутризародышевых впрыскиваний. Несмотря на то, что впрыскивания плазмы цесарки производились с 4-го по 18-ый день инкубации, нам не удавалось определить какой-нибудь период более чувствительный, для получения сближения, подобно тому, как его нашел Simmonsen (1956) для эритроцитов человека.

Степень иммунологического сближения по отношению к сывороточным белкам цесарки у цыплят, как и сближение по отношению к сывороточным белкам индейки у кур-эмбриональных парабионтов с индейкой, бывает ниже, чем степень сближения по отношению к чужеродным белкам, достигнутая у кроликов. Причина этой разницы коренится, вероятно, в видовых различиях в способности к иммунологическому сближению.

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# Ammonia Oxidation by *Nitrosomonas* Enzymes

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In studying the biochemical transformations caused by microorganisms two stages have as a rule to be passed. First the peculiarities of the process induced by living, multiplying microbes are established and only then an attempt is made to reproduce the same process without the aid of cells, i. e. by culture filtrates or enzyme preparations. In spite of the considerable progress made in the physiology and biochemistry of microbes, many transformations of carbon, nitrogen, sulphur and other elements have been followed up only in cultures of micro-organisms. So far, the enzymatic nature of a number of processes has not been proved. Occasionally new enzymes are described but the evidence is based upon experiments with resting microbe cells only. This is hardly justified, since there is no fundamental difference between experiments with microbe cultures and acute experiments with resting cultures. It is hardly justifiable to speak of enzymatic action unless the chemical process in question has been reproduced in a liquid free of microbes. The terms "extracellular" fermentation or "extracellular" oxidation, so often used previously do not meet the purpose as they emphasize that the chemical process occurs outside the cell. The term "cell free" fermentation would therefore be preferable.

Six years have elapsed since Vinogradsky's (1952) brilliant studies on nitrification. During this period our knowledge of the biology, occurrence and ecology of the nitrifying bacteria has been greatly supplemented with new data. However the chemistry of the nitrification process is still unclear. It is quite probable that ammonia oxidation to nitrites is accomplished through the agency of several enzymes but these are still unknown. The discovery of ammonia oxidation by the enzymes contained in *Nitrosomonas* cells would however have opened vast prospects for the study of the mechanism of nitrification. It is sufficient to mention the great role of cell-free alcohol fermentation in elucidating the chemistry of individual stages of this process.

Only one paper by Omeliansky (1953), has been published on nitrification in a medium free of *Nitrosomonas* cells. These cells were triturated with washed sea-sand, after which distilled water and ammonium sulphate were added to the suspension. No ammonia decrease was noted in these experiments as due to *Nitrosomonas* enzymes. Omeliansky therefore concluded that the chemical activity of these microorganisms cannot apparently be dissociated from their vital activity. It should be mentioned, however, that Omeliansky's procedure is not free from objections. His principal experiments were carried out with very old (4-month) *Nitrosomonas* cultures, the cells being dessicated prior to trituration over sulphuric acid, which should have affected the activity of the enzymes.

*Material and Methods*

The present studies were carried out with a pure culture of *Nitrosomonas europaea* isolated from soil. The methods of isolation and control of pure *Nitrosomonas* cultures have been described elsewhere-(Imshenetsky and Ruban 1952, 1953). It will be noted that subculture on meat—peptone broth—a procedure commonly used for checking the purity of nitrifying bacteria—cannot be recommended since lack of growth does not demonstrate the purity of these cultures. As a matter of fact, a number of bacteria are known which, although present in the *Nitrosomonas* culture, do not multiply on meat-peptone broth. These are mostly mycobacteria or myxobacteria. A very careful microscopic control of culture purity is required as some bacterial satellites, e. g. mycobacteria, are incidentally very similar in appearance to *Nitrosomonas*.

In the present study considerable amounts of nitrifying cultures were used, grown in large glass bottles of 15—20 l. capacity. Vinogradsky's medium was used, of the following composition: $(NH_4)_2SO_4$— 2.0 g.; $K_2HPO_4$ — 1 g.; $MgSO_4$ — 0.5 g.; NaCl — 2.0 g.; $FeSO_4$ — 0.4 g.; a mixture of microelements ($LiSO_4$, $CuSO_4$, $Al_2(SO_4)_3$, $SnCl_2$, $MnCl_2$, $NiCl_2$, $CoSO_4$, $TiCl_4$, KBr) — 1 ml.; distilled water — 1000 ml., carefully ground chalk — 1.0 g.; pH of the medium 7.2—7.4. A pure 16—20 day *Nitrosomonas* culture was used, grown in 20 ml. medium in Erlenmeyer flasks of 200 ml. capacity. After inoculation of the bottles, glass tubes were introduced 3—4 cm. from the bottom; these were used for aeration of the cultures with sterile air at a rate of 15—20 l. per minute. The air escaped through short glass tubes likewise passing through the corks and supplied with cotton filters.

The cultures were incubated 10—12 days at 22—24°. As a rule, the bacteria were cultivated in a set of bottles. Subsequently, the culture was filtered through a Seitz-filter, 13.5 cm. in diameter, with a membrane filter No. 3. The sediment was washed with sterile distilled water until the disappearance of traces of ammonia and nitrite. Then it was carefully ground for 30 minutes with sterile glass powder in a sterile agate mortar. Glass powder (Schott glass) in amounts of 0.25—0.40 g. were added per 10 g. of sediment (consisting of cells and salt crystals, mostly phosphates). 15 ml. sterile water was added per 10 g. of triturated sediment, the suspension agitated and incubated in a thermostat at 40° for 24 hours. During this period some autolysis of the nitrifying bacteria took place. The autolysate was then filtered through a small Seitz filter (35 mm. diameter with an asbestos filter SF to free it from the cells. Sterility of the filtrate was checked including for the nitrifying bacteria, by inoculating different nutrient media. The sterile autolysate was divided into two parts. In one of them, used as a control, quantitative colorimetric determinations of nitrites were made with the Griss reagents, of ammonia by distillation in vacuo, and of total nitrogen by the micro-Kjeldahl method. The second, experimental portion of the filtrate was incubated for five days in a thermostat at 37° after which similar determinations of nitrites, ammonia and nitrogen were carried out. A comparison of the results in the experimental and control parts indicate the quantitative changes undergone by ammonia and nitrite in the cell-free solutions.

As an additional control, filtrates of autolysates were used from cultures of heterotrophic organisms, viz. *Pseudomonas* sp., *Mycobacterium rubrum* and *Saccharomyces cerevisiae*. The filtrates were obtained by the same methods as those from nitrifying bacteria.

## *Results and Discussion*

### Decrease of Ammonia Content in Autolysates Obtained from *Nitrosomonas* Cells

Enzymatic oxidation of ammonia was studied on filtrates of autolysates of the *Nitrosomonas* cells. These were shown to be free of *Nitrosomonas* cells as well as of other bacteria. Quantitative ammonia determinations in the filtrates showed that they always contain $NH_3$. Hence no ammonium sulphate was added to the filtrates. Instead, the degree of oxidation by *Nitrosomonas* enzymes of ammonia already contained in the autolysates was ascertained. Ammonia and nitrite determinations were carried out in duplicate (30 determinations 15 experiments) (tab. 1). The data obtained may be summarized as follows: —

1. In the filtrates of autolysates, the nitrite content increases within five days on an average from 0.94 to 1.48 mg. N per litre.

2. The ammonia content of the autolysates greatly decreases, from 26.4 to 7.4 mg N per litre, i. e. by more than three and half times.

3. The amount of nitrites formed is much less than that of ammonia oxidized.

Table 1. Experiments with autolysate filtrates of *Nitrosomonas* cells without the addition of $(NH_4)_2SO_4$. (All figures are given in mg./l. Duration of experiment 5 days.)

| Exp. No. | Amount of ammonia | | | | Amount of $NO_2$ | | | | Decreased $NH_4$ | Formed $NO_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | initial | | final | | initial | | final | | | |
| | $NH_3$ | $N_2$ | $NH_3$ | $N_2$ | $NO_2$ | $N_2$ | $NO_2$ | $N_2$ | in mg. $N_2$ | |
| 1 | 38.0 | 31.3 | 35.0 | 28.8 | 4.0 | 1.2 | 8.8 | 2.7 | 2.5 | 1.5 |
| 2 | 25.0 | 20.6 | 14.7 | 12.1 | 0.2 | 0.06 | 0.3 | 0.1 | 1.5 | 0.04 |
| 3 | 3.0 | 2.5 | 0.1 | 0.1 | 0.7 | 0.23 | 2.5 | 0.8 | 2.4 | 0.57 |
| 4 | 16.2 | 13.3 | 10.0 | 8.0 | 1.1 | 0.3 | 2.0 | 0.6 | 5.3 | 0.3 |
| 5 | 17.6 | 14.5 | 10.0 | 8.0 | 1.1 | 0.3 | 2.3 | 0.7 | 6.5 | 0.4 |
| 6 | 28.0 | 23.0 | 0.2 | 0.1 | 0.7 | 0.23 | 0.8 | 0.26 | 22.9 | 0.03 |
| 7 | 28.0 | 23.0 | 0.2 | 0.1 | 0.9 | 0.3 | 1.1 | 0.36 | 22.9 | 0.06 |
| 8 | 3.7 | 3.0 | 2.0 | 1.5 | 8.3 | 2.5 | 12.5 | 3.8 | 1.5 | 1.3 |
| 9 | 45.2 | 37.2 | 26.6 | 21.9 | 0.7 | 0.2 | 1.1 | 0.3 | 15.3 | 0.1 |
| 10 | 0.9 | 0.7 | 0.12 | 0.1 | 4.3 | 1.3 | 5.6 | 1.8 | 0.6 | 0.5 |
| 11 | 54.4 | 45.2 | 32.3 | 26.6 | 2.3 | 0.7 | 3.6 | 1.1 | 18.6 | 0.4 |
| 12 | 51.9 | 42.7 | 0.12 | 0.1 | 0.6 | 0.2 | 4.5 | 1.5 | 42.6 | 1.3 |
| 13 | 1.1 | 0.9 | 0.9 | 0.8 | 1.9 | 0.6 | 5.9 | 1.6 | 0.1 | 1.0 |
| 14 | 108.8 | 89.6 | 4.0 | 3.3 | 9.8 | 3.0 | 10.7 | 3.3 | 86.3 | 0.3 |
| 15 | 61.6 | 48.4 | 0.035 | 0.03 | 9.8 | 3.0 | 10.7 | 3.3 | 48.37 | 0.3 |
| Average | 32.3 | 26.39 | 9.05 | 7.43 | 3.09 | 0.94 | 4.82 | 1.48 | 18.49 | 0.54 |

Such are the principal conclusions. Let us discuss certain details. Attention is drawn to the fact that the initial ammonia content of the autolysates varies greatly i. e. from 0.9 to 108.8 mg. per litre. This is apparently due to a number of causes, such as the degree of autolysis of *Nitrosomonas* cells, the initial ammonia content of the cells, the cultivation conditions, etc. However, in all 15 experiments the decrease of the ammonia content was quite regular. Not in a single experiment did ammonia increase above the control level or remain unchanged. The rate of ammonia oxidation varies from one experiment to another. Sometimes almost all ammonia is completely oxidized (experiments No. 3, 6, 7, 12 and 15). One can even speak of complete oxidation of ammonia. Thus, for example in exp. No. 12 the initial ammonia content decreased from 51.9 to 0.12 mg./l, and in exp. No. 16 from 61.6 to 0.035 mg./l. In some other experiments ammonia oxidation was much less intense.

The question arose as to whether the decrease in ammonia content of the autolysate is related to a change in the forms of nitrogen or to $NH_3$ volatility. Determinations were therefore made of total nitrogen of the filtrates of *Nitrosomonas* autolysates and the heterotrophic *Pseudomonas* sp., immediately on preparation and after five days incubation in a thermostat. The analyses were made according to Kjeldahl in a modification used for nitrogen determination in the presence of nitrites and nitrates. The figures obtained are summarized in table 2. It will be seen that the total nitrogen content does not change during the experiment. Thus, no ammonia is volatilized from the autolysates but oxidation of ammonia takes place under the influence of enzymes contained in the cells of nitrifying bacteria.

To prove the enzymatic nature of ammonia oxidation, the filtrates containing the enzyme systems were inactivated by high temperature i. e. boiled for different time intervals or autoclaved. A comparison of the ammonia content after five days in heated and non-heated filtrates indicates the degree of thermostability of the ammonia oxidizing systems. The results are summarized in table 3. It will be noted

Table 2. Total $N_2$ content of the autolysate filtrates of the cells of *Nitrosomonas* and *Pseudomonas* sp. ($N_2$ in mg./l.).

| Exp. No. | Microorganism | Amount of $N_2$ | | Average amount of $N_2$ | |
|---|---|---|---|---|---|
| | | initial | final | initial | final |
| 1 | *Nitrosomonas* | 85.6 | 86.6 | | |
| 2 | ,, | 109.2 | 111.6 | | |
| 3 | ,, | 103.9 | 102.69 | | |
| 4 | ,, | 108.49 | 109.6 | 98.35 | 98.86 |
| 5 | ,, | 84.56 | 85.8 | | |
| 6 | ,, | 98.4 | 96.9 | | |
| 7 | *Pseudomonas* sp. | 63.7 | 64.0 | | |
| 8 | ,, ,, | 136.9 | 135.7 | 100.07 | 100.25 |
| 9 | ,, ,, | 137.2 | 138.1 | | |
| 10 | ,, ,, | 62.5 | 63.2 | | |

Table 3. Effect of thermal treatment of cell-free autolysates of *Nitrosomonas europea* on ammonia oxidation.

| Duration of heating | Initial amounts | | After 5 days | | | |
|---|---|---|---|---|---|---|
| | | | $NO_2$ mg./l. | | $NH_3$ mg./l. | |
| | $NO_2$ mg./l. | $NH_3$ mg./l. | exp. | control | exp. | control |
| 30″ | 4.76 | 970 | 7.65 | 11.05 | 920 | 910 |
| | 2.22 | 372 | 2.3 | 2.7 | 264 | 231 |
| 1′ | 4.76 | 970 | 5.44 | 11.05 | 930 | 910 |
| | 2.22 | 372 | 1.44 | 2.7 | 267 | 231 |
| 2′ | 4.76 | 970 | 8.16 | 11.05 | 940 | 910 |
| 3′ | 4.76 | 970 | 7.86 | 11.05 | 930 | 910 |
| | 0.99 | 450 | 0.66 | 0.87 | 102 | 31 |
| 10′ | 1.62 | 250 | 2.64 | 3.102 | 0.0 | 0 |
| 15′ | 0.8 | 400 | 0.54 | 0.37 | 290 | 255 |
| | 14.0 | 292 | 14.08 | 11.88 | 220 | 225 |
| | 56.0 | 111 | 13.5 | 26.0 | 62 | 55 |
| 20′ | 5.9 | 295 | 5.06 | 4.73 | 230 | 195 |
| 30′ | 5.9 | 295 | 4.51 | 4.73 | 297 | 195 |
| | 56.6 | 66 | 10.0 | 26.0 | 65 | 30 |
| | 56.6 | 63 | 13.5 | 26.0 | 63 | 28 |
| | 25.8 | 361 | 20.0 | 36.4 | 358 | 234 |
| 60′ | 25.8 | 361 | 15.2 | 36.4 | 360 | 234 |
| | 56.6 | 475 | 6.7 | 26.0 | 469 | 236 |
| | 14.0 | 295 | 9.24 | 11.08 | 289 | 225 |
| * 10′ | 14 | 295 | 5.08 | 11.08 | 289 | 225 |
| 10′ | 14 | 295 | 7.04 | 11.08 | 291 | 225 |

* Autoclaved at 0.5 atm.

that in all control experiments there occurs a regular drop in the ammonia content, i. e. its oxidation through the agency of enzymes. A short, 5—10 minute boiling of the filtrates does not cause complete inactivation of the ammonia oxidizing enzymes. Boiling for 15—20 minutes causes a distinct inhibition of ammonia oxidation while complete inhibition is induced by boiling for 30 minutes and autoclaving

for 10 minutes at 0.5 atmosphere. In all experiments there invariably occurred a regular decrease of the filtrate ammonia although no considerable accumulation of nitrites took place. An inspection of tables 1 and 3 will show that the amount of nitrites formed in the filtrates is insignificant as compared with that of ammonia oxidized. It was suggested that a decrease occurs in the nitrite content of filtrates incubated in the thermostat. To check this the course of nitrite accumulation was investigated. The figures obtained are summarized in table 4. They confirm the previous conclusion as to ammonia oxidation and also show that on the second day the nitrite content of the filtrates is higher than on the fifth day. However, in these experiments the nitrite maximum is also lower than was expected. This fact may be accounted for in two ways: 1. Nitrite formation from ammonia is accomplished in several stages through the agency of several enzymes, the experimental conditions favouring the enzymes that oxidize ammonia but not those participating in further oxidation. 2. The nitrites formed interact with the amides contained in the autolysate filtrates and undergo a secondary reduction. Further enquiry is necessary to account for the lack of the expected quantitative ratio between oxidized ammonia and nitrites formed. It must be noted that such a correlation is likewise lacking in a pure developing *Nitrosomonas* culture.

To check the assumption that hydroxylamine is the intermediate product formed on oxidation of ammonia into nitrites, an attempt was made to oxidize hydroxylamine by cell-free filtrates. These data are summarized in table 5.

Table 4. The change in $NH_3$ and $NO_2$ content of cell-free *Nitrosomonas* autolysates (mg./l.).

| Exp. No. | Decrease of $NH_3$ | | | | | Initial | Formation of $NO_2$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | days | | | | | | days | | | | |
| | 1 | 2 | 3 | 4 | 5 | | 1 | 2 | 3 | 4 | 5 |
| 1 | 24 | 45 | — | — | 810 | 1.6 | 6.5 | 6.3 | — | — | 3.9 |
| 2 | 120 | 158 | 176 | — | 180 | 1.8 | 1.37 | 0.8 | 0.47 | 0.34 | 0.35 |
| 3 | 9 | 14 | 103 | 154 | 157 | 1 | 4.5 | 4.1 | 2.2 | 1.6 | 0.9 |
| 4 | 12.5 | 38 | 80 | 110 | 123 | 2 | 3 | 2.3 | 1.8 | 1.8 | — |
| 5 | 82 | 132 | 132 | — | 137 | 2.2 | 1.8 | 1.7 | 1.7 | 2.2 | 1.8 |
| 6 | — | — | — | — | — | 3.75 | 40 | 17 | — | — | 8.3 |
| 7 | 5.9 | 31 | 41.6 | — | 55 | 27.7 | 30.9 | 32.7 | 32 | — | 30 |
| 8 | 3 | 5 | 7 | — | 8 | 8 | 15 | 8 | 8 | — | 5 |
| 9 | + 72 | + 98 | + 148 | + 165 | + 155 | 1 | 1.3 | 1.2 | 1 | 1.5 | 1 |

N o t e: In experiment No. 9 the autolysate of *Mycobacterium rubrum* was used.

Tab. 5. Nitrite formation from hydroxylamine in cell-free autolysates of *Nitrosomonas europea* (in mg./l.).

| Exp. No. | Initial content of the autolysate | | Final $NO_2$ content of the autolysate | | $NH_2OH$ oxidized in water | Difference |
|---|---|---|---|---|---|---|
| | $NH_2OH$ | $NO_2$ | without $NH_2O$ | with $NH_2OH$ | | |
| 1 | 21.21 | 0.58 | 1.76 | 17.60 | 3.08 | 12.76 |
| 2 | 21.21 | 1.62 | 3.56 | 22.33 | 3.08 | 15.69 |
| 3 | 19.45 | 1.26 | 2.54 | 16.94 | 0.10 | 14.28 |
| 4 | 19.45 | 11.50 | 12.69 | 21.15 | 0.07 | 8.39 |
| 5 | 19.57 | 1.26 | 2.54 | 16.92 | 0.10 | 14.28 |
| 6 | 37.36 | 1.58 | 16.92 | 33.84 | 0.76 | 16.16 |

Tabl. 6. Experiments with autolysate filtrates of cells of heterotrophic microorganisms. (The figures are given in mg./l. Duration of experiments 5 days.)

| Exp. No. | Microorganisms | Amount of $NH_3$ | | | | Amount of $NO_2$ | | | | Increased $NH_3$ | $NO_2$ formed |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | initial | | final | | initial | | final | | | |
| | | $NH_3$ | $N_2$ | $NH_3$ | $N_2$ | $NO_2$ | $N_2$ | $NO_2$ | $N_2$ | | |
| 1 | *Saccharomyces cerevisiae* | 10.0 | 8.2 | 20.0 | 16.4 | 0.1 | 0.03 | 0.25 | 0.1 | 8.2 | 0.07 |
| 2 | ,, | 0.24 | 0.2 | 11.7 | 9.6 | 0.1 | 0.03 | 6.3 | 2.1 | 9.4 | 2.07 |
| 3 | ,, | 13.1 | 10.8 | 18.5 | 15.2 | 0.1 | 0.03 | 0.8 | 0.3 | 4.4 | 0.27 |
| 4 | ,, | 30.9 | 25.4 | 36.7 | 30.2 | 0.93 | 0.3 | 1.11 | 0.33 | 4.8 | 0.03 |
| 5 | ,, | 56.5 | 46.0 | 58.0 | 47.7 | 1.12 | 0.4 | 1.5 | 0.5 | 1.7 | 0.1 |
| 6 | ,, | 0.24 | 0.2 | 3.0 | 2.4 | 0.1 | 0.03 | 1.5 | 0.5 | 2.2 | 0.47 |
| 7 | *Pseudomonas* sp. | 0.24 | 0.2 | 10.2 | 8.4 | 0.1 | 0.03 | 0.6 | 0.2 | 8.2 | 0.17 |
| 8 | ,, | 5.4 | 4.4 | 6.0 | 5.0 | 0.1 | 0.03 | 0.1 | 0.03 | 0.6 | 0.00 |
| 9 | ,, | 50.19 | 41.3 | 54.31 | 44.7 | 1.88 | 0.63 | 2.19 | 0.7 | 3.4 | 0.07 |
| 10 | ,, | 62.5 | 51.5 | 77.0 | 63.4 | 1.0 | 0.3 | 1.2 | 0.4 | 11.9 | 0.1 |
| 11 | ,, | 42.0 | 34.5 | 54.4 | 44.7 | 0.1 | 0.03 | 0.93 | 0.3 | 10.2 | 0.27 |
| 12 | ,, | 94.3 | 77.7 | 108.8 | 89.6 | 0.25 | 0.1 | 0.45 | 0.2 | 11.9 | 0.1 |
| 13 | ,, | 53.6 | 41.2 | 80.2 | 66.1 | 1.2 | 0.4 | 1.2 | 0.4 | 24.9 | 0.00 |
| 14 | ,, | 48.1 | 39.9 | 70.9 | 58.4 | 0.6 | 0.2 | 1.5 | 0.5 | 18.5 | 0.3 |
| 15 | *Mycobacterium rubrum* | 10.0 | 8.2 | 32.0 | 26.2 | 0.5 | 0.16 | 1.31 | 0.4 | 18.0 | 0.24 |
| 16 | ,, | 0.24 | 0.2 | 2.1 | 1.7 | 0.10 | 0.03 | 1.73 | 0.6 | 1.5 | 0.57 |
| | Average | 29.84 | 24.37 | 40.23 | 33.10 | 0.52 | 0.17 | 1.41 | 0.47 | 8.73 | 0.30 |

It appears that hydroxylamine oxidation by the enzymes proceeds at a fairly high rate. It was additionally found that the capacity of cell-free *Nitrosomonas* autolysates to oxidize hydroxylamine is lost upon autoclaving—a fact which supports the suggestion as to the enzymatic character of oxidation. This capacity is specific for *Nitrosomonas* since it is lacking in the autolysates of the most diverse heterotrophic bacteria, such as *Mycobacterium rubrum, Myc. citreum, Sarcina lutea, Ps. fluorescens, Bac. mesentericus, Micrococcus rubifaciens, Proteus vulgaris, B. schützenbachi.*

The method of comparative physiology not only enables one to introduce some corrections into the results obtained, but also to confirm the specifity of the biochemical processes studied. The decrease in the ammonia content in the autolysates of nitrifying bacteria made it necessary to show that such a decrease does not take place in the autolysates of heterotrophic bacteria. The experiments were carried out with the cells of *Pseudomonas* sp., *Mycobacterium rubrum* and *Saccharomyces cerevisiae*. The methods of cultivation of heterotrophic microorganisms have been described above. The filtrates of autolysates were obtained in the same way as those of *Nitrosomonas*. The results obtained are presented in table 6.

The following conclusions are indicated by the figures of table 6: —

1. In the filtrates of autolysates obtained from the cells of heterotrophic microorganisms an increase occurs in the ammonia content by approximately 30% in five days. No decrease in ammonia, which is so characteristic for the autolysates of nitrifying bacteria, was ever noted. Nor did ammonia content remain unchanged in any of the experiments.

2. The autolysates of heterotrophic bacteria always contain nitrites but in a lesser concentration than those of nitrifying bacteria.

3. Within five days the nitrite content of heterotrophic autolysates increases by about two and a half times.

The above changes in the autolysates of heterotrophs are associated with a change in the forms of nitrogen since the total N remains constant throughout the experiment (table 3).

The above data show that there exists an enzyme which oxidizes ammonia. In this connection it seems valuable to discuss the results obtained by Omeliansky (1953) with *Nitrosomonas* cells. His attempts to oxidize ammonia from triturated cells proved futile and this might have been due to several reasons. Thus, the nitrifying cells were dessicated prior to trituration, which might have affected the enzyme activity. It will also be noted that the principal experiments were carried out with a very old culture (4-month-old) in which Omeliansky himself noted degenerating forms. The possibility is not excluded that *Nitrosomonas* cultures grown in flasks in such small volumes, cannot yield, a number of cells which would suit these purposes. It is also of importance that in these experiments a solution of ammonium sulphate was added, whereas ground *Nitrosomonas* cells, even if washed free of ammonia, are rather rich in $NH_3$ which is first oxidized. Nevertheless, Omeliansky's work was highly progressive, for at that time only a single oxidase laccase, was known, and there was almost no information as to the chemistry of the oxidation processes taking place in the bacterial cell. Experiments with heterotrophic microorganisms showed that on incubation of these autolysates in a thermostat, the ammonia content does not only fail to diminish but regularly increases. This is exactly what would be expected as in the autolysates desamination of amino acids occurs. Not only did the experiments on heterotrophs suggest that the drop in ammonia content of the *Nitrosomonas* autolysates is strictly specific but certain indications were obtained to the effect that in these autolysates two processes occur simultaneously. One of them is connected with ammonia oxidation resulting in a drop in its content, while the other should be accompanied by the accumulation of ammonia, as there are no grounds for negating the possibility of a parallel process of desamination. It is probable that proteins, peptides and amino acids do not in these autolysates undergo the same changes as those observed in the heterotrophic autolysates. If this be so, oxidation involves, not only ammonia contained in the autolysates at the beginning of the experiment, but also that formed in preserved autolysates. Hence it seems natural to conclude that the enzyme responsible for ammonia oxidation is sufficiently active (as is the case in some experiments), to oxidize all the ammonia almost completely. This assumption will probably be confirmed by determinations of various forms of nitrogen in the autolysates.

It appears quite plausible therefore that nitrification is an oxidation process consisting of several phases. At least two of them can already be indicated. The first consists in ammonia oxidation resulting in the formation of intermediate products which are similar to hydroxylamine, oximes and the like. These products are accumulated in the cells in appreciable amounts as evidenced by the capacity of nitrifying bacteria for endogenous nitrification, i. e. for the formation of nitrites in a medium devoid of ammonium salts. It is this initial enzymatic nitrification process that is particularly intense in the autolysates, i. e. transformation of ammonia into an intermediate oxidation product. The amount of ammonia oxidized is significant and hence a considerable amount of the intermediate product accumulates. The toxicity of hydroxylamine does not favour the suggestion that this substance is the intermediate oxidation product. Without foreshadowing the character of the

compounds formed on the oxidation of ammonia, the enzyme responsible for this oxidation may be spoken off as "ammon oxidase". The activity of this enzyme can readily be demonstrated by the above methods. Much less active is the second stage of nitrification, consisting in the oxidation of the intermediate product to nitrites. Cell-free nitrification results in insignificant nitrite formation as compared with the reduction of ammonia. It should however be pointed out that in liquid cultures of nitrifying microbe cells, the amount of nitrites formed is likewise much less than that of oxidized ammonia. The possibility is not excluded that the enzyme responsible for the second stage of oxidation requires different conditions for its activity than those required by ammon oxidase. It may also be suggested that the formation of nitrites is caused by an enzyme which is present not only in nitrifying bacteria but also in some other bacteria. This is indicated by the formation of nitrites in the autolysates of heterotrophic bacteria. These problems cannot be settled until further studies have been made.

*Summary*

1. The possibility of cell-free oxidation of ammonia by *Nitrosomonas* enzymes has been proved. The filtrates of the autolysates obtained from the cells of nitrifying bacteria contain both ammonia and nitrites. Within five days, the ammonia content of the filtrates decreases four times, eventually almost completely disappearing. The total nitrogen content of the filtrates does not change, hence the possibility of ammonia volatilization is excluded.

2. The enzymatic character of ammonia oxidation is confirmed by the fact that boiling for 30 minutes and autoclaving for 10 minutes inactivate the filtrates. Appreciable thermostability of the oxidative enzymatic systems of *Nitrosomonas* brings them close to peroxidases.

3. Oxidation of ammonia is accompanied by the formation of a rather insignificant amount of nitrites.

4. Cell-free filtrates obtained from autolysates of *Nitrosomonas* cells oxidize hydroxylamine to nitrites thereby confirming the theory according to which hydroxylamine is an intermediate product of ammonia oxidation.

5. The capacity to induce enzymatic oxidation of ammonia is specific for *Nitrosomonas*. Incubation of the filtrates of autolysates from cultures of various heterotrophic bacteria does not lead to a decrease, but rather to a considerable increase their ammonia content.

6. Oxidation of ammonia is apparently accomplished in several stages. At first ammonia is oxidized to intermediate products which in their turn are oxidized to nitrites. In filtrates free of *Nitrosomonas* cells the first stage is particularly active.

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# L-формы бактерий

## IV. Влияние температуры на развитие L-цикла у Proteus vulgaris и его значение для возникновения устойчивости к пенициллину

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Конечным звеном L-цикла развития бактерий является возврат к первоначальной бактериальной форме. Иными словами, в рамках L-цикла через различные переходные формы (крупные круглые тельца — ККТ, крупные продолговатые тельца — КПТ, элементарные тельца) восстанавливаются т. н. вторичные палочки. При изучении L-форм у Proteus vulgaris нас интересовало два вопроса: 1. осуществляется ли L-цикл при температурах ниже 37° С и при какой температуре еще осуществима регенерация; 2. не оказываются ли вторичные палочки более устойчивыми к пенициллину.

### Влияние температуры на развитие крупных круглых телец P. vulgaris под действием пенициллина

#### *Материалы и методика*

Мы пользовались одним штаммом Proteus vulgaris ($P_2$), а некоторые опыты дополнили сравнением с 3 штаммами P. mirabilis ($P_3$, $P_4$, $P_5$). Для посевов применялись 18—24-часовые культуры в бульоне, т. е. уже в стационарной фазе, что было удобно для наших опытов (образуется максимум ККТ).

Мы делали посевы на блоки агара, которые мы прикрывали стерильным покровным стеклышком и заливали в парафин. Питательная среда содержала 25% лошадиной сыворотки и пенициллин в концентрации 2, 6, 10, 20, 40 тысяч ед/мл. Контрольные препараты содержались в термостате при 37° С, опытные — при комнатной температуре (18—22° С) и в леднике при температуре от 0 до + 6° С при средней температуре около 4° С. Температура в леднике контролировалась ежедневно. Так как наблюдения препаратов производились при комнатной температуре, то для исследований при 4° С засевалось по меньшей мере 4 препарата, два из которых осматривались ежедневно, третий — через 7 дней, а последний — только в период образования вторичных палочек. И действительно, мы убедились, что в результате пребывания препаратов из ледника в течение нескольких минут ежедневно при комнатной температуре образование палочек ускорялось приблизительно на 12—24 часа. Мы делали снимки на кинопленку Foma-repro ortho II, обычно с помощью фазового контраста (кроме рис. 6).

#### *Результаты*

Рассмотрим развитие Proteus при исследуемых температурах и различных концентрациях пенициллина.

*2000 ед.* При сниженной температуре развитие ККТ протекало по существу так же, как и в контрольных препаратах (при 37° С). Главная разница заключалась в запаздывании развития, в общем пропорциональном снижению темпе-

ратуры (табл. 1). Другим отличительным признаком была склонность к образованию L-колоний (рис. 6) в контрольных препаратах, не наблюдавшаяся при комнатной температуре. При 4° С мы отметили несколько способов образования вторичных палочек (рис. 18):

а) Наиболее часто встречается отпочкование вторичных палочек на одном или на обоих полюсах КПТ (рис. 2). Это — самый распространенный способ и при более высоких температурах. Он был уже нами описан (Нермут и Нечас 1954);

б) менее часто встречается полярная фрагментация (Нермут и Нечас 1955), заключающаяся в постепенном распаде телец на все более и более мелкие фрагменты — вплоть до возникновения почти нормальных палочек;

в) последним способом является т. н. сегментация, когда тельце сначала разрастается в длину (в ширину оно бывает как толстое волокно и в течение роста еще больше утончается), а потом разделяется почти одновременно на нескольких местах на более короткие отрезки, — новые палочки.

*6000 ед.* В контрольных препаратах бросался в глаза быстрый распад ККТ на типичные скопления зернистости (рис. 3), из которых не развивались ни палочки, ни L-колонии. Зато при комнатной температуре здесь через 30 часов начиналась регенерация вторичных палочек подобно тому, как в леднике, но только там они появлялись через 6—7 дней. Распад ККТ осуществлялся и при 4° С, но во много раз медленнее и часто не полностью. Вокруг некоторых шаров бывали заметны мелкие крупинки, размерами около 0,4—0,6 $\mu$ (рис. 9). Случаи регенерации бациллярной формы бывали не так многочисленны, как у предшествующей концентрации пенициллина; исходными образованиями для них были немногочисленные КПТ (рис. 5).

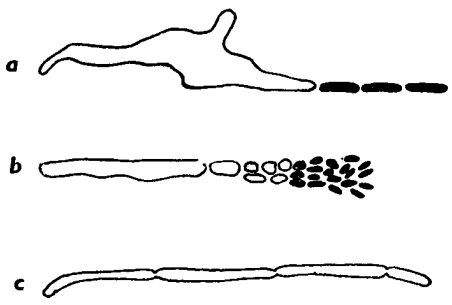


Рис. 18. a) Отшнуровка палочек на конце КПТ, b) полярная фрагментация, c) сегментация. (Схема.)

*10.000 ед.* В контрольных препаратах опять наблюдался быстрый распад ККТ. Только в отдельных случаях наступала еще регенерация палочек. В препаратах при комнатной температуре распад ККТ в гранулярные массы протекал гораздо умереннее и к 24-му часу во многих случаях наступала регенерация бациллярной формы из КПТ. У штаммов $P_2$ и $P_4$ через 10 и 17 дней вырастали отдельные L-колонии, из которых после их перенесения в среду без пенициллина в анаэробных условиях возникали палочки. На рис. 17 показаны волокна, вырастающие из мелких и крупных шариков, взятых из L-колоний. Через 6 часов из этих продолговатых телец образовались многочисленные вторичные палочки.

При 4° С распад ККТ осуществлялся чрезвычайно медленно, и только через 7—9 дней образовались КПТ, а из них потом через 10—14 дней — новые палочки (табл. 2). Первая генерация вторичных палочек обычно по форме несколько отличается от нормальных бактерий: они бывают обычно толще и с более заостренными концами, что в нормальной культуре никогда не встречается, хотя Proteus, как известно, принадлежит к микробам, в значительной степени полиморфным.

*20.000 ед.* В контрольных препаратах наблюдался всегда быстрый (в течение 6—8 часов) распад ККТ в шарики различной величины. Ни с L-колониями, ни с регенерацией мы не встречались. Лишь в одном случае из 10 мы добились

регенерации палочек, но только после пересева на свежую питательную среду и в анаэробных условиях.

При комнатной температуре способность к регенерации у всех штаммов сохранялась долго. Так, у штамма $P_2$ регенерация вторичных палочек наступала всегда через 3—4 дня, у штамма $P_5$ через 10 дней вырастали L-колонии, которые на среде без пенициллина снова давали палочки. У остальных штаммов через 17 дней палочки выросли на среде без пенициллина при аэробных условиях. Культивация при анаэробных условиях давала отрицательные результаты.

В холодильнике через 12—14 дней образовались КПТ, а из них, начиная с 19—23-го дня — новые палочки. Из этого видно, как долго сохраняется жизнеспособность клеток бактерий (или их фрагментов) при пониженной температуре.

*40.000 ед*. При такой высокой концентрации мы при всех температурах наблюдали распад ККТ в типичные мелкие скопления шариков, или же образование зернистых шаров, которые постепенно распадались. Следует подчеркнуть, что при этой концентрации процент ККТ бывает значительно меньше (Нермут и Нечас 1956). Поэтому в препаратах преобладают палочки, по большей части пустые, с отчетливо видимой зернистостью. Эти палочки с течением времени также распадаются. В примокультурах мы никогда не наблюдали регенерации. В одном случае при комнатной температуре мы нашли у штамма $P_5$ 4 L-колонии типа 3 А, из которых только в аэробной субкультуре выросли колонии Proteus.

Характерным результатом высоких концентраций пенициллина (начиная с 6000 и выше) является распад ККТ, который начинается очень скоро, в особенности в тепле. Следует подчеркнуть, что он проявляется неравномерно, т. е. поражает не все шары одновременно и при 37° С бывает сильнее и быстрее, чем при комнатной температуре или в холодильнике. Рис. 4 иллюстрирует неравномерность развития ККТ: некоторые ККТ представляются еще однородными, другие — вакуолизированными, а остальные распадаются в мелкую или крупнозернистую массу. Некоторые шары оказываются довольно крупными (около 0,8 $\mu$) и, по нашим наблюдениям, в течение дальнейшего развития могут еще больше увеличиваться (до 1—2 $\mu$, рис. 12) или вакуолизироваться (дня через 2—3 после распада ККТ). Вакуоли вначале бывают совсем небольшими, позднее значительно увеличиваются (рис. 13, 14). Так как зернистость располагается обычно небольшими группами, то часто и из вакуолизированных шаров возникают гроздьевидные образования, напоминающие иногда (и по размерам) сильно вакуолизированные ККТ (рис. 11). Вакуоли обычно бывают окружены остатками первоначальной плазмы в виде каймы или небольшой почки (рис. 15). Аналогию возникновения вакуолей в шарах можно видеть в т. н. L-организмах, где мы наблюдали подобное явление после перенесения элементарных телец на новую среду: у изолированных телец обычно начиналась вакуолизация (рис. 16).

Подводя итоги отдельных опытов, можно сказать, что снижение температуры вызывает прежде всего замедление развития ККТ, которое ясно выражено при 4° С и менее выразительно, разумеется, при комнатной температуре. Замедление отражается в общем благоприятно на регенерации бациллярной формы: там, где при температуре в 37° С ККТ быстро распадались, не наблюдалось обычно восстановления вторичных палочек, тогда как при той же концентрации пенициллина, но более низких температурах — наступала регенерация. В некоторых случаях мы наблюдали (при комнатной температуре) возникновение L-колоний даже и при таких высоких концентрациях, как 20 и 40 тысяч ед/мл, что никогда не наблюдалось при 37° С. Можно утверждать, что комнатная температура является наиболее благоприятной для восстановления бациллярной формы Proteus.

Другим типичным результатом низких температур является склонность к росту в длину. Мы никогда не наблюдали случаев возникновения вторичных палочек из ККТ путем т. н. центральной фрагментации, так что практически единственным элементом регенерации при пониженной температуре являются КПТ (кроме мелкой зернистости — элементарных тесец, — представляющих основу L-колоний).

*Резюме*

Мы исследовали развитие крупных круглых телец (ККТ) Proteus vulgaris и P. mirabilis при температуре в 22° и 4° С и при воздействии различных концентраций пенициллина (2, 6, 10, 20 и 40 тыс. ед/мл).

1. При комнатной температуре мы наблюдали полный и неполный L-циклы. Прерванный цикл пока нам не встречался.

2. При 4° С наблюдался только неполный цикл.

3. При исследуемых температурах распад ККТ и регенерация вторичных палочек с понижением температуры замедляются.

4. Способность ККТ (или же крупных продолговатых телец) образовать палочки при пониженной температуре сохраняется дольше, чем при 37° С, даже при более высоких концентрациях пенициллина.

## Значение L-цикла бактерий для возникновения устойчивости к пенициллину

Для выяснения вопроса устойчивости вторичных палочек в пенициллину мы поставили следующие опыты: 1. пассажи культуры Proteus vulgaris в жидких и на плотных средах с пенициллином; 2. определение устойчивости вторичных палочек, выросших из одного общего ККТ; 3. определение содержания пенициллина в культуре P. vulgaris в бульоне.

*Методика*

Определение чувствительности палочек Proteus к пенициллину производилось по методу серийных разведений (3-часовой культуры). Мы делали пассажи или в сывороточном бульоне (25%), или на блоках агара с 2000 ед/мл (анаэробно). Содержание пенициллина мы определяли или с помощью иодометрии, или по методу линейной диффузии по Пешеку (1952).

*Результаты*

С помощью пассажей в жидкой среде не были получены однозначные результаты. После 6 пассажей не наблюдалось сколько-нибудь заметного повышения устойчивости к пенициллину.

При следующих опытах мы делали пассажи Proteus на плотной среде с пенициллином и при ежедневном микроскопическом контроле отмечали процент образования ККТ через 2 часа после посева, а также время и способ регенерации вторичных палочек. Мы исходили из того предположения, что у более устойчивых палочек процесс регенерации из ККТ должен протекать быстрее и что через определенное время палочки вообще должны перестать образовать ККТ. Мы произвели в общем 27 пассажей. Опишем сжато, как общая картина реакции на пенициллин менялась в течение пассажей. Вначале, при первом пассаже, 94% палочек реагировало образованием ККТ. Регенерация осуществлялась обычным способом через 30 часов. При 19-ом пассаже около 40% палочек образовало ККТ и регенерация тянулась 4 дня. При последнем пассаже ККТ образо-

вались только в 5% случаев, а регенерация не была отмечена ни на одном препарате. Поэтому мы и не могли уже определить по плану устойчивость вторичных палочек к пенициллину после 30-го пассажа. Запаздывание регенерации графически представлено на рис. 19. Начиная с 9-го пассажа, почти на всех препаратах встречались L-колонии (типа 3 Б), часто служившие единственным источником

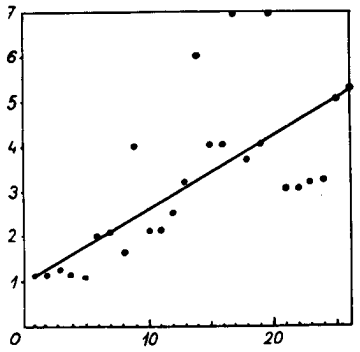


Рис. 19. Продление периода регенерации вторичных палочек из ККТ в течение пассажей на плотной среде с пенициллином. Ось X: количество пассажей, ось Y: время регенерации в днях.

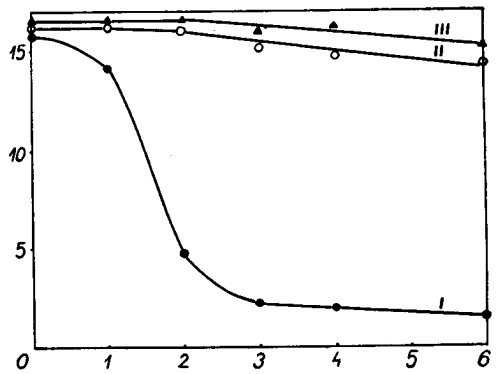


Рис. 20. Влияние температуры на падение уровня пенициллина в физиологическом растворе. Ось X: количество дней, ось Y: количество ед. пенициллина в 1 мл в тысячах. I кривая: 37° С, II кривая: 22° С, III кривая: 4° С.

вторичных палочек. Начиная с 21-го пассажа, L-колонии на некоторых препаратах уже не появлялись, а регенерация часто наблюдалась только на 1 из 3 препаратов. Все эти явления очень напоминали картину, наблюдавшуюся при воздействии гораздо более высоких доз пенициллина (6—40 тыс. ед/мл — Нермут и Нечас 1956). Таким образом, и этот опыт не свидетельствовал о повышении устойчивости вторичных палочек под влиянием многократного контакта с пенициллином.

Поэтому мы приступили к определению устойчивости вторичных палочек, возникших из ККТ при прямом микроскопическом контроле. Из общего количества приблизительно 100 изоляций нам 52 раза удалваось получить вторичные палочки из одного ККТ, и определить у них чувствительность к пенициллину. В 37 случаях она соответствовала контролю, в 3 случаях была выше (на 1 пробирку), а в 12 случаях — ниже. Во всех случаях несоответствия контролю была отмечена некоторая разница в помутнении разведения культуры в сравнении с контролем. Вначале мы не придавали этому значения, но опыты показали, что их результаты в значительной степени зависят от количества клеток. Устойчивость колебалась в границах от 500 до 2000 ед/мл. Оказалось, таким образом, что устойчивость вторичных палочек не повышается сколько-нибудь существенно. Метод разведений для определения устойчивости не исключает возможности некоторого повышения устойчивости. Но, как известно, незначительное повышение не должно быть обязательно специфическим, оно может вызываться неточно отмеренными дозами для посева и т. п. Мы потому и придерживались этого метода, что он, благодаря своей неслишком большой чувствительности, исключал эти неспецифические отклонения. Необходимо еще подчеркнуть, что при всех использованных нами до сих пор концентрациях пенициллина (от 7 до 40 тыс. ед/мл) мы всегда встречались с образованием ККТ и что при концентра-

циях в 2000 ед/мл и выше мы никогда не находили палочек, которые размножались бы прямо, не проходя L-цикла. Поскольку некоторые палочки не обрззоваали ККТ, они отмирали и распадались.

Если вторичные палочки не устойчивы к пенициллину, это означает, что содержание пенициллина в период регенерации должно быть значительно ниже, чем в начале опыта. Если это так, то необходимо установить причину его умень-

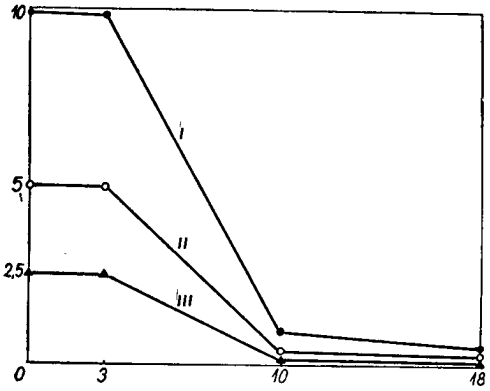


Рис. 21. Падение уровня пенициллина в бульоне при 37° С. Ось Х: количество дней, ось У: количество единиц пенициллина.

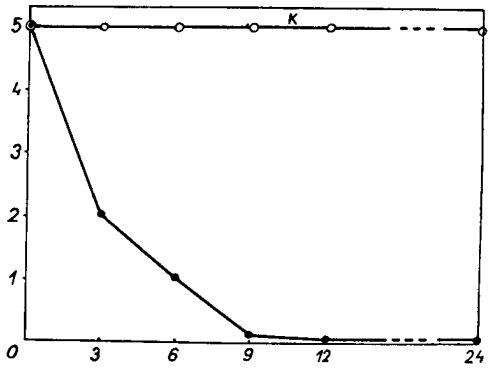


Рис. 22. Падение уровня пенициллина в культуре Proteus vulgaris при 37° С. Ось Х: время в часах, ось У: количество единиц пенициллина на 1 мл в тысячах. Кривая К: контроль.

шения. Поэтому мы исследовали сначала, как падает количество пенициллина в жидкой среде в связи с температурой. При первых же опытах оказалось, что падение содержания пенициллина при pH 5,5—6,0 в значительной степени зависит от температуры (рис. 20): в термостате (при 37° С) титр пенициллина с первоначальных 16.000 ед/мл упал в течение 6 дней до 1800 ед/мл, т. е. на 88,8%; при комнатной температуре наблюдалось падение с 16.200 ед/мл на 14.400 ед/мл (на 11,2%), а в леднике (4—6° С) с 16.500 на 15.200 ед/мл (на 8%). Эти показатели были установлены иодиметрическим путем.

При другом опыте мы с помощью диффузионного метода определяли падение содержания пенициллина в бульоне. Как показывает рис. 21, характер кривой здесь практически отвечает кривой при 37° С на рис. 20. Из этого вытекало бы, что в период регенерации палочек (через 24—30 час.) содержание пенициллина все еще было бы очень высоким, что противоречило бы результатам наших предшествовавших опытов. Поэтому мы принуждены были проследить изменения уровня пенициллина в среде, засеянной 18-часовой культурой P. vulgaris. При нескольких первых опытах мы определяли первоначальное и конечное содержание пенициллина, т. е. его содержание в период, когда была отмечена регенерация палочек. В период регенерации его содержание бывало всегда ниже, чем 0,03 ед/мл, т. е. практически равнялось нулю. Чтобы выяснить ход кривой падения содержания пенициллина в культуре, мы отмечали содержание пенициллина через каждые 3 часа вплоть до 36-го часа (рис. 22). Контрольная кривая за исследуемый отрезок времени практически совсем не снижается, тогда как опытная кривая падает очень быстро (табл. 3). В этот период (с 3-го по 12-ый час) культуры состояли преимущественно из шаров средних размеров (около 5—10 $\mu$), а около 10% составляли палочки или их остатки. Палочки не бывали одно-

родными и не проявляли признаков роста или размножения. Регенерация вторичных палочек начиналась только с 24-го часа и полного развития достигала к 30-му часу, т. е. начиналась через 12—15 часов после падения содержания пенициллина до суббактериостатических величин. Эти результаты очень важны и свидетельствуют о значительной способности ККТ расщеплять пенициллин на его инактивные составные части.

*Резюме*

1. Вторичные палочки, регенерирующие из ККТ Proteus vulgaris, не бывают более устойчивыми к пенициллину, чем первичные (исходные).

2. Содержание пенициллина в жидкой среде в период регенерации практически равняется нулю. Регенерация начинается приблизительно через 12 час. после падения содержания пенициллина до этого уровня.

Автор полагает, что L-цикл бактерий не имеет какого-нибудь особого значения для возникновения штаммов, устойчивых к пенициллину.

*(Табл. XIX, XX, XXI).*

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# L-forms of Bacteria

## IV. The Influence of Temperature on the Development of L-cycle of *Proteus vulgaris* and its Significance for the Development of Resistance to Penicillin

M. NERMUT

*Summary*

The final link in the L-cycle of bacteria is their return to the original bacterial form. In other words, "secondary" rods regenerate within the L-cycle from various preceding forms (large bodies, long bodies, elementary bodies). When studying L-forms of *Proteus vulgaris*, two questions were of particular interest: 1. Whether the L-cycle would take place at temperatures lower than 37° C and at what other temperatures could regeneration of secondary rods take place, and 2. Whether these secondary rods were more resistant to penicillin than the original rods.

### The Influence of Temperature on the Development of Large Bodies of *Proteus vulgaris* Produced by Penicillin

Work was carried out with strains of *P. vulgaris* and *P. mirabilis* using the method of agar blocks (anaerobic) at temperatures of 4—6° C, 22° C and 37° C. The concentration of penicillin was 2, 6, 10, 20 and 40 thousand units/1 ml.; the medium contained 25% equine serum. Phase contrast photography was carried out with a cine-film Foma repro ortho II.

Table 1.

| 2,000 units PE/ml. | Formation of LRB | Regeneration of secondary rods | Remarks |
|---|---|---|---|
| 37° C | 2 hrs. | 24—30 hrs. | |
| 22° C | 4— 6 hrs. | 24—48 hrs. | Occasionally after 15 hrs. |
| 6°—8° C | 8—24 hrs. | 5— 7 days | Comm. 5th day, ends 7th day |
| 4 °C | 16—30 hrs. | 8—10 days | Comm. 8th day, ends 10th day |

The results are summed up in part in tab. 1 and 2. A decrease in temperature leads primarily to a slowing down in the development of large bodies. This is very clearly expressed with a temperature of 4° C and is—understandably—less evident at room temperature. Slowing down is seen to be propitious on the whole for regeneration of the bacillary form. In cases where the large round bodies rapidly disintegrated at a temperature of 37° C, regeneration of secondary rods did not, as a rule occur, whereas it occurred at lower temperatures, with the same concentration of penicillin. In some cases the development of L-colonies (at room temperature) was observed with concentrations of penicillin as high as 20 and 40 thousand units/ml., whereas at 37° C this never occurred. It may therefore be said that room temperature is the best for regeneration of the bacillary form of Proteus.

A further typical feature of lower temperatures is a tendency to elongated growth. The development of secondary rods from large bodies by central fragmentation was

Table 2.

| Temp. | Conc. PE in thous. | Formation of LRB | Disintegration of LRB | Formation of LLB | Regeneration of rods | Formation of L-colonies |
|---|---|---|---|---|---|---|
| 37° C | 10 | 2 hrs. | 8—24 hrs. | — | 24 hrs. | — |
| | 20 | 2—4 hrs. | 8—24 hrs. | — | — | — |
| | 40 | 6—12 hrs. | 12—24 hrs. | — | — | — |
| 22° C | 10 | 4 hrs. | 1—3 days | 12 hrs. | 24 hrs. | 17 days |
| | 20 | 4 hrs. | 1—3 days | 24 hrs. | 3—4 days | 10 days |
| | 40 | 8 hrs. | 2—5 days | — | — | 10—14 days |
| 4 °C | 10 | 8—36 hrs. | 3— 5 days | 7—9 days | 10—14 days | — |
| | 20 | 1—4 days | 6—10 days | 12—14 days | 19—23 days | — |
| | 40 | 1—4 days | 7—14 days | — | — | — |

never observed, so that at low temperatures the large long bodies are virtually the only regenerative element (apart from the small granules, or elementary bodies, which form the basis of the L-colonies).

What is the significance and position of the large long bodies in the individual types of the L-cycle of the bacteria? In the first place, what is their relationship to the large round bodies? It cannot be said that they are identical with the large round bodies, for reasons of difference in form and function. The large round bodies are for the most part spherical in shape, whereas the large long bodies are elongated, usually bipolar and sometimes tripolar. Sometimes they are thin and very long, sometimes thicker and shorter (figs. 1 and 2). They are a further stage in the development of the large round bodies, a stage capable of regeneration. The large majority of large long bodies regenerate into rods. That cannot, however, be said of the large round bodies. It is known, for example, that with higher concentrations of penicillin the large round bodies disintegrate. On the other hand, development of elementary bodies from large long bodies was never observed, only from large round bodies. Large long bodies, therefore, are one of the possible stages in the development of the large round bodies and according to our experience develop more frequently under aerobic conditions than under anaerobic conditions and at low temperatures rather than at high temperatures (i.e. 37° C) and also in the presence of certain growth substances (e.g. from yeast cells). They occur most frequently in an incomplete L-cycle, less frequently in a complete cycle and never in an interrupted cycle.

## *Conclusion*

A study was made of the development of large round bodies of *Proteus vulgaris* and *P. mirabilis* at temperatures of 22° C and 4° C under the influence of various concentrations of penicillin (2, 6, 10, 20 and 40 thousand units/ml.).

1. At room temperature a complete and incomplete L-cycle were observed. An interrupted cycle has so far not been observed.

2. At 4° C only an incomplete cycle was observed.

3. At the temperatures investigated disintegration of the large round bodies and regeneration of the secondary rods takes place more slowly, in direct relationship to the fall in the temperature.

4. Ability of the large round bodies (or of the large long bodies) to produce rods is maintai ned for a longer period, and with higher concentrations of penicillin, at temperatures lower than 37° C.

## The Significance of L-cycle of Bacteria for the Development of Resistance to Penicillin

Investigation of the question of resistance of secondary rods which regenerate in the course of a complete or incomplete L-cycle is important for comprehending the biological significance of L-forms of bacteria. Troitsky and Pershina (1950, cited by Málek 1951) are of the opinion that the new rods are more resistant to penicillin than the original rods.

Other authors especially Dienes and Weinberger (1951), Joiris (1955) and Schnauder (1955) take the view that the secondary rods are not more resistant to penicillin than the primary rods. An attempt was made, on our own material, to throw light on this question. The following experiments were carried out: 1. Determination of the resistance of secondary rods which regenerated from one large body, 2. Observation of the penicillin level in a broth culture of Proteus up to the time of regeneration of the secondary rods.

Large bodies from a broth containing 1000 units of penicillin were isolated by the Lindner drop method and regeneration of the rods was observed under the microscope. The resistance of these rods to penicillin was then determined by the dilution method in Lahelle's modification (1948). The results were read after 5—7 days and resistance ranged from 500—2,000 units/ml. Most authors read results within 24 hours and that is why their resistance values are so much lower. We, however, found that the titration result is stabilised only after the third, or even fifth, day of incubation and that reproducible results can only be obtained in this way. From a total number of 100 cells isolated, secondary rods were obtained in 52. In 37 cases resistance to penicillin was the same as in the control culture, in three cases it was higher (by one degree) and in 12 cases it was lower. In all the divergent cases a slight difference in the inoculation dose had been recorded (technical failure).

Table 3.

| Hours | Level of PE in culture of *P. vulgaris* in units/ml. | Level of PE in bouillon (control) in units/ml. |
|---|---|---|
| 0 | 5,000 | 5,000 |
| 3 | 2,000 | 5,000 |
| 6 | 1,500 | 5,000 |
| 9 | 100 | 5,000 |
| 12 | 3 | 5,000 |
| 24 | 1 | 5,000 |
| 36 | <0,03 | 5,000 |

The level of penicillin in the broth was determined iodometrically or by Pešek's method of linear diffusion (1952). The following considerations were taken into account:

If the secondary rods are not resistant to penicillin, this must mean that in the period of regeneration the level of penicillin is considerably lower than at the commencement of the experiment. If that is the case, it is necessary to seek the cause of its decrease. We therefore first studied the fall in the level of penicillin in the fluid medium under the influence of temperature. In the first experiments it was seen that the fall in the penicillin level was markedly dependent on temperature at a pH of 5.5—6.0 (fig. 20). In the thermostat (37° C) the penicillin level dropped in six days from the original value of 16,000 units/ml. to 1,800 units/ml., i. e. by 88.8%, whereas at room temperature it fell from 16,200 units to 14,400 units (by 11.2%) and in the refrigerator (4°—7° C) from 16,500 units to 15,200 units (by 8%). These values were determined iodometrically.

It follows from these results that at the time of regeneration of the rods (24—30 hours) the penicillin level is still very high, but this contrasts with our previous results. We therefore observed changes in the penicillin level in a medium inoculated with an 18-hour-old culture of *P. vulgaris*. In the first few experiments the initial level and the final level (i. e. when regeneration of the rods was found) were determined. In the period of regeneration, the level was always lower than 0.03 units, i. e. virtually zero. In order to obtain information on the decrease of penicillin in the culture, the penicillin level was observed every three hours up to 36 hours (fig. 22). During this period the control curve virtually does not fall at all, whereas the experimental curve falls rapidly (tab. 3). During this time (3—12 hours), the cultures were composed mainly of round bodies of medium size (about 5—10 $\mu$) and about 10% consisted of rods or their residue. The rods were not homogenous and showed no signs of growth or proliferation. Regeneration of secondary rods began from 24 hours and was at its height at about 30 hours. It began, therefore, 12—15 hours after the penicillin level had dropped to subbacteriostatic values. These results are very interesting and indicate that the large round bodies are in large measure capable of breaking down penicillin into its inactive components.

*Conclusion*

1. Secondary rods which regenerate from large round bodies of *Proteus vulgaris* are not more resistant to penicillin as compared with primary rods (i. e. the original rods).

2. The level of penicillin in the fluid medium at the time of regeneration of the secondary rods is practically zero. Regeneration commences approximately 12 hours after the penicillin level has dropped to this value.

The author is of the opinion that the L-cycle of bacteria is not of especial significance for the development of strains resistant to penicillin.

*(Tables XIX, XX, XXI).*

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# Induction of Bacteriophages by Ultraviolet Light in a Naturally Polylysogenic Strain of *Staphylococcus aureus*

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The effect of ultraviolet (UV) light on bacteria has already been the subject of detailed analysis from many aspects. In recent years the induction of bacteriophage formation by lysogenic bacteria, discovered by Lwoff, Siminovitch and Kjeldgaard (1950) and elaborated for various species of bacteria, in particular by Lwoff (1951 etc.), Weigle and Delbrück (1951), Jacob (1950 etc.) and by other authors has come to form an independent branch of this subject. Induction in lysogenic bacteria has been also studied by Rosenberg (1956), and this subject was dealt with by Herčík (1953).

The increase in the spontaneous production of bacteriophages by lysogenic strains (induction) by irradiation of cultures of these with UV light has therefore already been investigated in detail. Only two authors, however, have so far dealt with the influence of UV light on the production of phages by strains which produce two types of phage simultaneously; these are Jacob (1952, 1954) and Welsch (1953). Both these authors first of all attempted experimentally to find an answer to the question of whether every polylysogenic cell produced both phages when lysed, or whether certain cells always produce only one type. Jacob, who used artificially cultured double lysogenic strains of *P. pyocyanea*, arrived at precise, though not altogether conclusive results. Welsch's work with a naturally double lysogenic strain of *Micrococcus pyogenes aureus* did not provide a clear answer to the problem.

An attempt was therefore made to resolve this problem along independent lines, following on from the results obtained in earlier experiments with lysogenic staphylococci (Rosenberg and Šmarda 1955). Staphylococcal strains are frequently naturally polylysogenic, as described recently by Gorrill and Gray (1956). The work was carried out with a naturally polylysogenic strain of *Staphylococcus aureus haemolyticus*.

## *Methods*

Previously described strains of the species *Staphylococcus aureus haemolyticus* (Rosenberg and Šmarda 1955) were used in the experiments, in particular the lysogenic strain LS 2 and the sensitive strains CS 1, CS 4, CS 10, CS 13 and CS 14. Before commencing the experiments, all the strains were propagated at least five times from one colony and further cultivated in a similar manner at least once a week. In the case of the strain LS 2, endogenous lysogeny was demonstrated serologically according to the method of McKinley (1925).

The strain LS 2 now produces basically two types of bacteriophage, which differ both biologically (in the lytic spectrum of the series of sensitive strains) and also serologically. These are phage F 4, for which the specific indicator strain is CS 4 and F 13, the specific indicator strain of which is C 13. (This means that F 4 does not lyse C 13 or vice versa.) F 4 is present in the filtrate of a 6-hour culture of LS 2 in an average titre of $10^4$ and F 13 in an average titre of $10^5$. Phage 1 can also, however, be demonstrated in filtrate of LS 2; this is identical with F 13, except that it also, to a low degree, lyses CS 4. A clone

of CS 4 which was sensitive only for F 4 and not for F 1 could not be isolated; every one of the seven clones of CS 4 which were cultured out lost its sensitivity for F 4 at the same time as for F 1. It is probable therefore, that a small number of phages in filtrates of LS 2 in these experiments formed plaques both on CS 4 and on CS 13.

The following media were used: meat-peptone broth enriched with Difco proteose-peptone and yeast hydrolysate, and 2% peptone agar, also containing Difco proteose-peptone. The pH of both media was 7.4.

The bouillon cultures of LS 2 were always irradiated at the commencement of the log phase of growth, i. e. following 4½ to five hours' incubation, in a cylindrical container of transparent quartz glass with an internal diameter of 22 mm. and walls 1.5 mm. thick. The distance of the low-power UV lamp, the full spectrum of which was used, was 50 cm. from the slowly and regularly rotating container (about 45 r.p.m.). Unless otherwise stated, irradiation was carried out in the laboratory in daylight, or in mixed day and electric light. After exposure lasting for 1'15", 2'30", 5', 7'30", 10', 12'30", 15' and 17'30", the same volume of culture was always removed and stored under the same lighting and temperature conditions until irradiation of the rest of the culture had been completed. All specimens were then incubated together in the dark for 30 minutes at 34—37° C.

After incubation they were filtered through collodion ultrafilters of medium porosity about 650 mμ. (The same batch of filters was used for all specimens in the same experiment.) The filtrates were diluted serially with bouillon or with 0.6% physiological saline until a concentration was reached which was suitable for counting the plaques, i. e. up to $10^{-4}$. (These staphylococcal phages cause only very slight clearing of the bouillon cultures and can be titrated only according to the production of plaques on agar.) Small amounts (usually 0.1 ml.) of the filtrates were transferred by micropipette to agar plates freshly inoculated with sensitive strains. When dry, the plates were incubated at 34—37° C. The results were always read off the next day. The titres were calculated to a % of the titres of non-irradiated controls.

*Results*

The experiments were based on the preliminary finding that a filtrate of LS 2 contains phages which consistently lyse the bacterial strains CS 1, 4, 10, 13 and 14. Filtrates of specimens of an irradiated culture of LS 2, removed from the culture at given intervals during exposure, were tested after diluting on one to three of the above sensitive strains simultaneously. The results of 30 such experiments were evaluated, i. e. an average of six experiments per strain.

After plotting on a graph the percentual increases in the titres of the phages carried by the individual sensitive strains for the various times of exposure, roughly two types of curve were obtained (fig. 1). The first gave approximately the development of the titres of phages on CS 1 and 4, and the other on strains CS 10, 13 and 14. Both curves indicate a high degree of induction of phage production by LS 2. At the same time, however, the induction of phages forming plaques on strains CS 1 and 4 is not in general so great as that shown by the three other indicator strains. It can, to a certain extent, be judged from the curves that the titre of phages produced spontaneously in higher quantities by the culture, will be increased by induction even more than the titre of phages, which are liberated spontaneously in lower quantities. The second type of curve (CS 10, 13 and 14) shows a second peak after exposure for 17'30", which is higher than the first one occurring after 10 minutes. (In the case of CS 1 and 4, the filtrates were not tested after this period of exposure.)

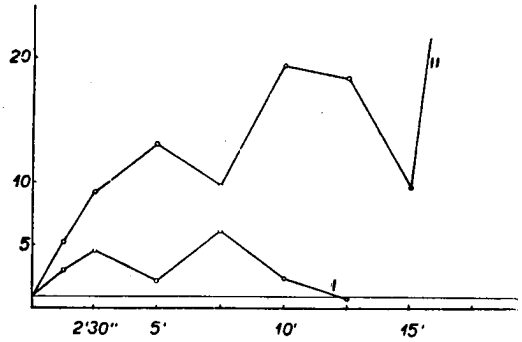


Fig. 1. Arithmetical average of titres of phages forming plaques on CS 1 and 4 (curve I) and on CS 10, 13 and 14 (curve II), in the course of induction of LS 2, as compared with the controls. $x$: time of exposure in minutes, $y$: titre as a % of control: 100.

The higher values of phage titres found after irradiation of a culture of LS 2 with UV light, as illustrated in fig. 1, cannot, however, be evaluated simply as the result of induction. Under the given conditions, the titres of the phages are also influenced by a whole series of other factors, including in particular the following: inequality of the cells in the stock cultures of LS 2, from which a strain was taken in these experiments (Málek 1955), duration of growth of the culture in the log phase during the experiment, and hence variability of the number of cells in the specimens, the sublethal or lethal effect of UV light on the cells, adsorption of released phages back on to the cells or debris, reactivation of induced cells by visible light and inactivation of phages by UV light, together with a whole series of further factors participating in filtration and also in testing the filtrates on the indicator strains. (Of these factors, inactivation of liberated phages by UV rays can be excluded to a certain extent, at least as far as exposures up to 12 minutes are concerned. It is known that bouillon markedly absorbs UV light. Irradiations of filtrates of a culture of LS 2 were carried out several times with exposure intervals up to 12 minutes, under the same conditions, as irradiations of the culture itself with and without visible light, and no real decrease in the titre of any of the phages was found.)

The effect of actual induction cannot be separated from the majority of factors mentioned above. No further attention was therefore paid to evaluating it quantitatively and statistically; we just confirmed the basic finding that the production of phages by the strain LS 2 can be greatly induced by UV light. It was decided, however, in the further work to study the interrelationship of the titres of the individual types of phages liberated by LS 2 during irradiation. It was assumed that the above factors which complicate the effect of induction on the absolute titre of the phages would have approximately the same effect on all the phages in the specimen and would not therefore fundamentally affect the relationship of the titres of the individual phage types. The curves in fig. 1 indicated that this relationship would not be constant in the course of induction.

In this part of the work, 30 irradiation experiments were carried out, using the lysogenic strain LS 2 and the indicator strains CS 4 and CS 13. Irradiation was carried out as described above, except that exposure was carried out for intervals of 3, 6 and every further three minutes up to 30 minutes. After dilution, every specimen was plated with the same micropipette on the two indicator strains. In these experiments a modification of d'Hérelle's titration method (1926) was used, based on the author's own experience and consisting chiefly in greater accuracy of the count.

The filtrates were diluted by progressively adding 0.1 ml. filtrate to 0.9 ml. 0.6% physiological saline. The cultures used in the experiment were not always of the same concentration and the initial titre of the phages could not, therefore, always be the same. Their relationship on a given medium, however, was constant.

The proportion of the titres of F 13 and F 4 in $4^1/_2$—5-hour cultures of LS 2 was constant, varying only very slightly from the ratio of 3 : 1, regardless of the absolute height of the titres, which were different in the individual experiments. In the statistical evaluation, this ratio was seen to be statistically significant (standard error of the difference of both averages = 1). In the course of induction the titre of F 13 increased by as much as 400 times, the maximum being reached after 15 and 27 minutes' exposure, while the titre of F 4 increased at most 12 times, with the maximum again after 15 and 27 minutes' exposure. The development of the ratio of the titre of F 13 to the titre of F 4 in the course of induction is best seen if the value of the titre of F 4 in every specimen is taken to equal 1. This relationship then reached the ratio of 367 : 1.

As was expected, the development of the titres of the phages was not always the same. The chronological course of the increase in the titres varied in the different experiments. The maximum increase was therefore often obtained after different doses, while the values of the increase in the individual experiments were also different. In one experiment, the titre of F 4 actually showed no increase on irradia-

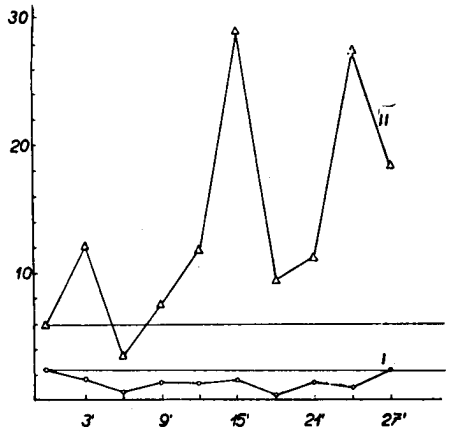


Fig. 2. Development of titre of F 4 (I) and F 13 (II) in the course of induction of LS 2. Experiment carried out 16. 1. 1956. $x$: time of exposure in minutes, $y$: titre: $10^4$.

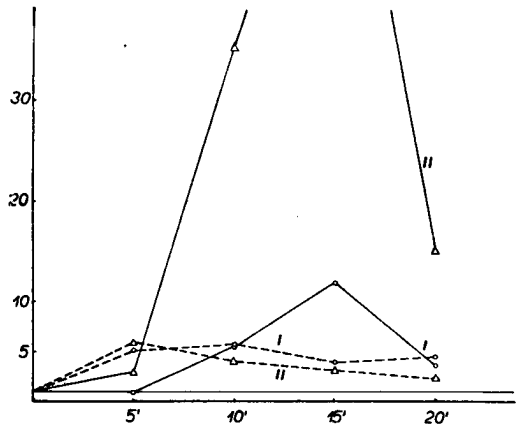


Fig. 3. Development of titre of F 4 (I) and F 13 (II) in the course of induction of LS 2, in the presence of visible light (full lines) and without (interrupted lines). Experiment carried out 7. 6. 1955. $x$: time of exposure in minutes, $y$: titre as a % of control: 100.

tion of LS 2, while the titre of F 13 rose 4.9 times (fig. 2). No attempt was therefore made to give a statistical evaluation of the individual periods of exposure. In all experiments without exception, however, and whatever the time of exposure, the increase in the titre of F 13 was always far greater than in F 4, i. e. lysis of the cells to F 13 was induced far more than lysis to F 4.

The first induction maximum after about 15 minutes' exposure was followed, as a rule, by a somewhat steep drop in the titre of both phages, evidently as a result of the relative preponderance of adsorption of liberated phages on to bacteria and bacterial débris over further phage production by the cells. It is interesting to note that in many experiments—as in the first part of the work—a further sharp increase in the titres occurred after 20—30 minutes' exposure, which usually reached a peak higher than the first peak at 15 minutes. This phenomenon can be explained by sudden lysis of many cells as a result of the large dose of UV light, at a moment when the increasing lethal action of the UV light reached a certain equilibrium with the action of induction. (The possibility of such an interpretation is also borne out by the conclusion of Franklin 1954, that lysogenic bacteria which have been inactivated by UV light can still liberate bacteriophage.)

A similar method was used in four consecutive experiments in which the course of induction during irradiation in the dark was studied. It is interesting that in these experiments completely different results were obtained. The titre of F 13 attained at most six times the value of the control, while the titre of F 4 increased as much as 34 times. The most effective time of exposure for the induction of F 4 and F 13 was 5—6 minutes. An example is given in fig. 3, which shows the curves of the titres of both phages in two experiments carried out consecutively under exactly the same

conditions, except that in one irradiation was carried out in the daylight and in the other in the dark.

As compared with the experiments in the preceding series, the effect of induction in the dark is seen to be almost always far less than in the light, and as far as the proportional increase in the titres of F 4 and F 13 is concerned, a greater increase was found in the titre of F 4 than in that of F 13.

*Discussion*

After irradiating the lysogenic staphylococcal strain LS 2 with UV light, which damages the normal course of nucleoprotein metabolism in its cells (in the presence of visible light), a considerable increase occurred in the number of particles of both phages produced by the strain. They did not increase proportionately, however, but the titre of F 13 increased far more than that of F 4. It could, of course, be assumed that the increase in the ratio of the titre of F 13 to F 4 was due to production of both phages in changed proportion by every individual lysing cell. Even if this could be assumed to be partially the case, the increase in the proportion mentioned in all the experiments is so great that such an explanation alone is not sufficient and the possibility must be admitted that as a result of induction many cells lyse while liberating F 13, without simultaneous production of F 4.

This does not, of course, exclude the possibility that after induction a whole series of different types of cell disintegration appear in the culture. Some cells may disintegrate without forming phage (Hradečná 1952, Herčík 1953), others may simultaneously produce both F 4 and F 13 in varying proportion and it is even possible that some produce only F 4. The important fact, however, is that almost certainly many produce only F 13. The proportion of titres of the two phages in an irradiated culture, considered from this aspect, would then represent some kind of section through this scale of forms of disintegration, among which lysis into F 13 predominates.

A study of the results of these experiments permits no definite conclusions as to the normal, spontaneous manner of production of the two phages by a growing culture of LS 2. It is possible that it is analogous to the one described above. At all events, the ability to produce both phages in the proportion of 3 : 1 is genetically bound to every cell of the strain, since a clone isolated from any cell again produces the phages in this proportion. Nevertheless, it is very probable that during lysis some cells produce only one phage.

In resolving the problem of whether one induced polylysogenic bacterium can produce both types of phage, Welsch (1953) used the "single burst" technique, i. e. the study of lysis of isolated induced bacteria. After making a statistical analysis of the results, he came to the conclusion that the question could not be decided by this method.

Jacob (1952) states that weak doses of UV radiation result in the liberation of one type of phage by one cell, while strong doses induce the simultaneous development of both types of phage by one bacterium. The present author takes the view that the experiments from which Jacob arrived, mathematically, at these conclusions, on the basis of the conception of probacteriophage (Lwoff 1953, Jacob 1954), do not take sufficiently into account the whole dynamics of a lysogenic culture subjected to irradiation during growth. Jacob's general view on this subject is that in a culture of a strain which produces two phages, the development of which can be induced, each phage, after induction, can be produced by given cells which do not at the same time produce the other phage. The present author agrees with Jacob.

The results of the present experiments also concur with Jacob's finding that with high doses of UV irradiation the effect of induction on the production of phage decreases. It is assumed that this is a manifestation of the lethal effect of irradiation on the cells themselves, of progressive sterilization of the culture and of a preponderance of adsorption of formed phage particles over production of further phages by the surviving cells.

The differences in the results of irradiation in the presence of light and in the dark are also explained in the same way. In the dark, the bacteria were evidently inactivated far more by the same doses, induction was masked by inactivation and the effect of induction was therefore displayed far more weakly in the titre of the phages. (The somewhat lower production of F 13 than of F 4 indicates more rapid inactivation of the cells producing them.) Induction was still displayed to some extent with the lowest doses, after which there was no further increase in the number of phages and the number of liberated mature phages decreased by adsorption, the decrease being approximately the same in both types. In visible light, in which photoreactivation evidently asserted itself through the interaction of low-energy photons, induction was much more effective.

*Summary*

1. The formation of both types of bacteriophage liberated by the lysogenic staphylococcal strain LS 2 can be induced to a marked degree by UV light.

2. The constant quantitative proportion of 3 : 1 in which these two phages are produced in all passages by a culture of LS 2, is disturbed as a result of induction, production of one of the phages being increased by as much as 400 times, and of the other, with the same dose, twelve times at the most. The one phage is therefore probably liberated by cells which do not produce the other phage simultaneously, although potentially every cell can produce both.

3. The quantitative effect of induction is displayed differently if the culture is irradiated in the presence of visible light, which reactivates the cells, than if it is irradiated in the dark.

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# УФ-индукция бактериофагов у естественно полилизогенного штамма Staphylococcus aureus

Я. ШМАРДА

*Резюме*

Повышение продукции лизогенными штаммами бактериофагов в результате облучения культур этих штаммов ультрафиолетовыми лучами (УФ), — т. е. индукция, — подробно исследовалось уже целым рядом авторов. Но только два из них занимались также вопросом влияния УФ-лучей на продукцию фагов штаммами, которые производят одновременно два типа фага, Jacob (1952, 1954) и Welsch (1953). Оба они попытались экспериментально ответить на вопрос возможности продукции двух различных фагов одной и той же подвергшейся индукции клеткой. Jacob, который пользовался искусственно выращенными дважды лизогенными штаммами P. pyocyaneus, пришел в этом вопросе к определенным, хотя и не вполне убедительным результатам. Работа же Welsch-a с естественно дважды лизогенным штаммом Staphylococcus aureus в этом направлении не принесла ясного ответа.

В своей работе мы попытались приблизиться к решению этой проблемы собственным путем, исходя из своих предшествовавших опытов с лизогенными стафилококками (Розенберг и Шмарда 1955). Мы пользовались собственным полилизогенным штаммом Staphylococcus aureus haemolyticus.

Этот штамм (LS 2) производит, по существу, 2 типа бактериофага, отличающиеся друг от друга как в биологическом, так и в серологическом отношениях: фага F 4, для которого специфически чувствительным является штамм CS 4, и фага F 13, для которого специфически чувствительным является штамм CS 13. Фага F 4 штамм производит в среднем в титре $10^4$, а F 13 в среднем в титре $10^5$. Однако не исключено, что некоторое (небольшое) количество фагов в фильтратах LS 2 в наших опытах образовало негативные колонии как на CS 4, так и на CS 13.

Мы облучали культуры штамма LS 2 в бульоне в начале логарифмической фазы их роста в цилиндрическом сосудике из прозрачного кварцевого стекла, который медленно вращался. Мы пользовались полным спектром УФ-лампы низкого давления с расстояния в 50 см, при доступе видимых лучей. После отдельных экспозиций (от 1 до 30 мин.) из сосудика брался всегда одинаковый объем культуры, который хранился до окончания облучения при таких же условиях, при каких остаток культуры еще облучался. Потом все образцы одновременно инкубировались в темноте при 34—37° С в течение 30 мин. После

инкубации все образцы фильтровались через коллодийные ультрафильтры со средним диаметром пор около 650 mμ. С помощью бульона или физиологического раствора из фильтрата приготовлялись серийные разведения возрастающего ряда (вплоть до концентрации $10^{-4}$), которые титровались по количеству негативных колоний на отдельных тест-культурах на агаре. Полученные титры вычислялись в % по отношению к титрам необлучавшихся контрольных штаммов.

Мы исходили из предварительно полученных данных о том, что фильтрат LS 2 содержит фаги, неизменно вызывающие лизис штаммов CS 1, 4, 10, 13 и 14. На этих 5 чувствительных штаммах мы испытывали фильтраты отдельных образцов облучавшейся нами культуры LS 2. Была произведена оценка в общем 30 опытов. Нанеся на график % повышения титров фагов, который показали отдельные чувствительные штаммы — в зависимости от продолжительности экспозиции, — мы получили 2 типа кривых (график 1). Титры фагов на CS 1 и 4 менялись приблизительно по первой кривой, а фагов на CS 10, 13 и 14 — по второй. Обе кривые показывают высокую степень индукции образования фагов штаммом LS 2. При этом однако индукция фагов, образующих негативные колонии на штаммах CS 1 и 4, в общем не достигает такой высоты, как индукция фагов, появляющихся на 3 остальных штаммах. На основании кривых можно также судить, что титр тех фагов, которых уже сама культура производит больше, возможно с помощью индукции повысить больше, чем титр тех фагов, которых самопроизвольно образуется меньше. Интересно, что на второй кривой (CS 10, 13 и 14) после экспозиции в течение 17 мин. 30 сек. обозначается второй максимум, еще более высокий, чем первый (через 10 мин.).

Не следует однако расценивать приведенное на графике 1 повышение титров фагов после УФ-облучения культуры LS 2 как результат одной только индукции: на титр фагов при данных условиях опыта влияет еще целый ряд факторов, как неравноценность клеток в запасных культурах LS 2, откуда производились пересевы (Малек 1955); продолжающийся в течение опыта рост культуры в логарифмической фазе, а вместе с тем и изменения количества клеток в образцах; сублетальное и даже летальное действие УФ-лучей на клетки; обратная адсорбция выделяемых фагов клетками или их детритом; реактивация подвергшихся индукции клеток под действием видимого света и инактивация фагов под действием УФ-лучей, — кроме ряда других факторов, проявляющихся при фильтрации и при тестах с фильтратами. (Изо всех этих побочных влияний можно исключить, — по крайней мере при экспозиции до 12 мин., — только УФ-инактивацию выделяемых фагов, которых в этом случае в совершенстве защищает бульон.) Действие собственно индукции невозможно отделить от влияния большинства этих факторов. Поэтому мы не производили точной количественной оценки ее действия и удовлетворились в этом отношении принципиальным констатированием факта, что УФ-лучи в весьма сильной степени индуцируют продукцию фагов штаммом LS 2.

Далее мы исследовали взаимное отношение титров отдельных типов фагов, выделяемых в течение облучения штаммом LS 2. Мы исходили из предложения, что вышеупомянутые факторы, осложняющие действие индукции на титр фагов, влияют приблизительно одинаково на всех фагов в образце, т. е. не оказывают существенного влияния на соотношение титров отдельных типов фагов. Кривые на графике 1 показывают, что это соотношение в ходе индукции не постоянно. В этой части работ мы произвели опять 30 опытов облучения, для которых мы пользовались лизогенным штаммом LS 2 и чувствительными штаммами CS 4 и CS 13. Каждое разведение образца наносилось всегда на оба чувствительных штамма.

Культуры, бравшиеся для опыта, не всегда бывали одинаково густыми, поэтому и исходный титр фагов F 13 и F 4 не мог быть всегда один и тот же. Однако в данной среде их соотношение бывало постоянным и колебалось (незначительно) около величины 3 : 1 (независимо от абсолютных значений титров). Была доказана статистическая значимость этого соотношения. В ходе индукции титр F 13 повышался и в 400 раз, с максимумом при экспозиции в течение 15 и 27 мин., а титр F 4 — не больше, чем в 12 раз, с максимумом также при экспозиции в течение 15 и 27 мин. Развитие соотношения между титрами F 13 и F 4 в ходе индукции становится наиболее наглядным, если принять значение титра F 4 в каждом образце за единицу: это соотношение доходило и до 367 : 1.

Как мы и предполагали, развитие титров фагов протекало не всегда одинаково: кривая повышения титров в отдельных опытах проходила по разному; максимальное повышение титра получалось после различных доз облучения и бывало неодинаково в отдельных опытах. При одном опыте титр F 4 даже вовсе не повышался, тогда как титр F 13 увеличился в 4,9 раз (график 2). Поэтому мы не пытаемся дать статистическую оценку действия отдельных экспозиций. Но при всех опытах без исключения и при любой экспозиции, при данных условиях опыта, повышение титра F 13 бывало гораздо значительнее, чем повышение титра F 4. Таким образом, распад клеток на фаги типа F 13 индуцировался гораздо интенсивнее, чем распад на фаги типа F 4.

После первого максимума индукции около 15-ой мин. экспозиции следовало обычно довольно резкое падение титров обоих фагов, обусловленное, по-видимому, относительным преобладанием адсорбции уже сформировавшихся фагов на бактерии или их детрит — над дальнейшей продукцией фагов клетками. Интересно, что при многочисленных опытах, — как и в первой части работы, — после экспозиции в течение 20—30 мин. наблюдалось новое резкое повышение титров, обычно даже превышающее первый максимум (около 15-ой минуты). Это явление мы объясняем внезапным распадом большого количества клеток под влиянием значительной дозы УФ-лучей в момент, когда усиливающееся летальное действие облучения до известной степени уравновешивало индукцию.

Для оценки влияния фотореактивации на исход облучения мы в 4 опытах исследовали индукцию при облучении в темноте. При этих опытах титр F 13 превышал показатели контроля не более, чем в 6 раз, тогда как титр F 4 повышался и в 34 раза. Наиболее эффективная для индукции F 4 и F 13 продолжительность экспозиции бывала 5—6 мин. В качестве примера приводим на графике 3 кривые титров обоих фагов, полученные при 2 опытах, поставленных непосредственно один за другим при совершенно одинаковых условиях. При первом из них мы производили облучения при дневном свете, при втором же — в темноте. Как видно из опытов, эффективность индукции в темноте бывает меньше, а что касается соотношения повышения титров F 4 и F 13, наблюдается более значительное повышение титра F 4, чем F 13.

Значительное увеличение отношения титра F 13 к титру F 4 после УФ-индукции при доступе видимых лучей трудно объяснять как изменение соотношения, в котором отдельные распадающиеся клетки производят обоих фагов. Повышение этого соотношения во всех случаях настолько значительно, что приходится допустить, что многие клетки под влиянием индукции распадаются на F 13, не производя одновременно F 4. Мы допускаем, конечно, что после индукции в культуре встречается целый ряд различных типов распада клеток. Некоторые клетки могут распадаться вообще без образования фага (Градечная 1952, Герчик 1953). Возможно, что самопроизвольное образование обоих фагов

протекает аналогично. Во всяком случае, способность образовать оба типа фага в сооотншении 3 : 1 генетически связана с каждой клеткой штамма.

В вопросе развития в результате индукции в лизогенной культуре двух типов фагов, которые возможно индуцировать, мы присоединяемся ко взгляду Jacob-a (1952), что после индукции каждый из них может производиться определенными клетками, не производящими одновременно второго фага.

Разницу результатов облучения при доступе видимых лучей и в темноте мы объясняем реактивирующим действием видимых лучей. Повидимому, в темноте бактерии теми же дозами облучения инактивировались гораздо значительнее, и поэтому действие индукции отражалось на титре фагов гораздо слабее, и только при наиболее низких дозах облучения.

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# The New Antibiotic BU 271

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The following large-molecular actinomycetic antibiotics have been described in the literature: micromonosporin (Waksman et al. 1942), actinomycetin (Welsch 1937—1947), antibiotic 2377 (British patent 719.230) and thermomycin (Schöne 1951). These antibiotics can easily be distinguished from the small-molecular antibiotics by means of dialysis through a cellophane membrane. Micromonosporin and actinomycetin are of a protein character, antibiotic 2377 is not a protein, but gives a positive anthrone reaction for carbohydrates, while in the case of thermomycin no details are given as to the character of the molecule.

The new antibiotic, BU 271, was obtained from a strain of soil actinomycetes listed under the number 271. On agar media, colonies of this actinomycete have a whitish mycelium with yellowish spores. Underneath, the mycelium is rust colour. Pigment is not diffused.

When cultured on a shaker apparatus, an isolated strain of the actinomycete produced 43 streptomycin units/ml. The composition of the medium was as follows: glucose 1%, starch 1.5%, corn steep 0.5%, $(NH_4)_2SO_4$ 0.35%, NaCl 0.5%, $CaCO_3$ 0.5%. When inoculated on agar media, subcultures with a maximum production of 460 strep. units/ml. were obtained. On further reinoculation on to potato agar slants, however, production of the antibiotic fell rapidly.

A second batch gave 20 subcultures, about half of which (9) did not produce the antibiotic on the shaker, but of which about a quarter (5) gave very satisfactory maximum production (about 1,000 s. u./ml.), which was not, however, very stable. Considerable differences were observed between the original cultures and the subcultures on individual days of submersion cultivation, and production of the antibiotic fluctuated considerably in the same strain when this was inoculated in different weeks, the cause being probably poor stability of the strain.

On further distribution of a selected, highly productive strain (about 1,000 s. u./ml. on cultivation on a shaker), 28 subcultures were obtained, all of which produced the antibiotic on culturing on the shaker. About half the subcultures gave very satisfactory production (1,000—2,000 s. u./ml.), but the latter still fluctuated quite considerably, even when the strain gradually became stabilized. More stable strains were obtained only after further inoculation, when evaluation on agar plates showed no great differences in the size of the inhibition zones in the individual subcultures.

Antibiotic 271 is stable only at low temperatures. At 2° C it remains stable for a number of weeks. At laboratory temperature it is rapidly destroyed in the presence of an acid, neutral or basic pH, even after only 24 hours. On boiling for 15 minutes it is destroyed in all pH values tested (2.0, 7.0, 9.0).

The antibiotic is more effective in the presence of an alkaline reaction (pH 7.5 to 8.0); with 50% blood serum its efficacy is reduced by half. It is destroyed by organic solvents. Crude preparations of the antibiotic were obtained by lyophilization of dialyzed filtrates of a culture of the actinomycete.

The antibiotic is not dialyzed through a cellophane membrane and its spot on a paper electrophoregram stains with bromphenol blue, indicating that the molecule is probably of a protein character.

Antibiotic BU 271 acts particularly on Gram-positive bacteria. It usually acts on various species of Gram-positive bacteria in concentrations of 1—8 units/ml., but does not act on species of Gram-negative bacteria even in concentrations of 100 units/ml. It has been found to act especially strongly on *Mycobacterium phlei* and in a comparison with other bacteria its action on this species was greater than that of streptomycin.

The antibacterial spectrum and large molecule of antibiotic BU 271 resemble those of micromonosporin (Waksman et al. 1942, Welsch 1947), but it differs from this antibiotic by its stability, method of isolation (it is not precipitated on adding 50—90% ethanol or acetone) and by its coloration. If a filtrate of a micromonosporin culture is heated for 60 minutes to 100° C, about two thirds of the antibiotic are destroyed, whereas on heating a solution of BU 271 to 100° C, the whole of the antibiotic is destroyed in 15 minutes. Micromonosporin also has an orange pigment, while BU 271 is colourless.

On intravenous administration of a preparation with a strength of 1,150 units/mg., $LD_{50}$ in mice was 45 mg./kg., corresponding to 1,035 units/mouse.

We are indebted to L. Novotný of the Institute of Chemistry of the Czechoslovak Academy of Science for the crude preparations of the antibiotic.

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# Новый антибиотик BU 271

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*Резюме*

Антибиотик BU 271 был получен из точнее нами не определявшегося вида почвенного лучистого грибка, обозначенного № 271. Антибиотик устойчив при 2° С в течение нескольких недель, но при лабораторной температуре быстро разрушается (уже через 24 часа), при кипячения в течение 15 мин. разрушается полностью. Антибиотик более эффективен при алкалической реакции (pH 7,5 — 8,0). 50% сыворотка крови ослабляет его действие на половину. Органические растворители его разрушают. Антибиотик это бесцветное вещество, не диализирующее через пленку целофана. Пятно антибиотика на бумажной электрофореграмме окрашивается бромфеноловым синим. Антибиотик действует преимущественно на грам-положительные бактерии. На Mycobacterium phlei он действет сильнее, чем стрептомицин. По своему антибактерийному спектру и крупной молекуле он близок к микромоноспорину, от которого отличается однако по степени устойчивости, по способу изоляции (он не осаждается после прибавления этанола или ацетона) и по окраске. $LD_5$, для мышей при введении в вену бывала 45 мг/кг.

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# The New Antibiotic BU 306

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Among the antibiotics of a protein character which are recovered from actinomycetes and act on Gram positive and Gram negative bacteria, only actinomycetin has been described in the literature (Welsch 1937—1947).

A new antibiotic, BU 306, was obtained from a strain of soil actinomycetes, listed in our collection under the number 306. Colonies of the actinomycete, which are rusty brown underneath, form yellowish spores on normal agar media; the pigment is not produced into the medium.

In submerged cultivation on a shaker, on the medium described for antibiotic BU 271 (Ševčík et al. 1956), the original strain of actinomycete produced 120 streptomycin units/ml. On setting up cultures on agar media of the same composition, generations with a maximum production of about 1,000 s. u./ml. were obtained. On progressive transfer to potato agar slants, production of the antibiotic decreased from 1,000 to 300 s. u./ml. Following fresh culture on an agar medium, 35 colonies were obtained, most of which (27) produced the antibiotic, but on a smaller scale (100—400 s. u./ml.). These cultures were somewhat labile. The further transfer of a culture producing 400 s. u./ml. gave 32 colonies, in seven of which production was much higher (1,000—1,600 s. u./ml.).

Cultures of the actinomycete of BU 306 were set up on an agar medium containing corn steep (0.5%), the amino acid content of which provides a suitable environment on potato agar slants in a refrigerator at + 4° C.

A high yield of the antibiotic (1,500—2,000 s. u./ml.) was obtained on culturing on a reciprocal shaker on the medium containing corn steep described above. In some cases a maximum production of 3,500 s. u./ml. was obtained, after 6—7 days' cultivation.

On fermenting in a glass laboratory tank with a capacity of 1,000 ml., a maximum production of 1,150 s. u./ml. was obtained after 120 hours' culturing. The laboratory tank was inoculated with a 24-hour-old vegetative inoculum from the shaker (2% of the medium volume). Mixing was carried out at 375 revs./minute, aeration one vol./minute.

At 2° C, the antibiotic remains stable for a number of weeks. It is destroyed by boiling. It is also destroyed by organic solvents.

The antibiotic BU 306 does not dialyze through a cellophane membrane. After being separated from ballast substances on the paper electrophoregram, the spot of antibiotic stains with bromphenol blue, indicating the possibility that the molecule is of a protein character.

Unrefined samples of the antibiotic, with a strength of 2,000 units/mg. were prepared by means of lyophilization of dialyzed filtrates of a culture of the actinomycete.

Using the diffusion method of microbiological titration, BU 306 is most effective at a pH of 8.0. Its effectiveness decreases along with a fall in the pH. Its activity

is reduced by half by 50% blood serum. BU 306 acts on Gram positive and Gram negative bacteria. It acts on the micro-organism *Mycobacterium phlei* in approximately the same concentrations as streptomycin.

BU 306 is not very toxic. A concentration of 1,000 units/ml. produced no toxic effect on the protozoa *Tetrahymena gelei* and *Euglena gracilis* (culture filtrate). Toxicity in mice depended on the amount of high-molecular ballast substances present and not on the concentration (degree of effectiveness) of the antibiotic itself. In intravenous injections, $LD_{50}$ was 0.5 mg./20 g. in a lyophilized preparation, following dialysis, with a strength of 2,000 units/ml. In preparations with a strength of 800 units/mg., $LD_{50}$ was 0.7 mg./20 g., corresponding to 560 units/mouse, and in a preparation with 120 units/mg., $LD_{50}$ was 1 mg./20 g., corresponding to 120 units for mouse.

BU 306 also had an effect on Ehrlich's ascitic tumour in mice. In a patch test, in which the tumour cells were placed for three hours in a refrigerator together with the antibiotic, the mice survived on the administration of doses of 125 units/mouse. While the control mice, which were inoculated with the tumour, without the antibiotic, died in 12—15 days, the mice which were given the antibiotic survived for over a month. For this experiment a preparation with a concentration of 1,000 units/mg. was used. Similar results were recorded in therapeutic tests with the same concentration of the antibiotic, in which the latter was administered once a day for a total of eight days. The antibiotic was injected intraperitoneally, the first dose being administered three days after inoculation with the tumour.

Among the high-molecular antibiotics (of a protein character), which act on Gram positive and Gram negative bacteria, only actinomycetin is known from among the actinomycetes. BU 306, however, differs from actinomycetin by its stability and by the method of its recovery. BU 306 is not precipitated either by acidification to a pH of 3—4, using HCl, or by adding four volumes alcohol or acetone.

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## Новый антибиотик BU 306

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*Резюме*

Антибиотик BU 306 был получен из точнее не определенного лучистого грибка, обозначенного нами № 306. В сыром виде препарат антибиотика представляет желтоватое нейтральное вещество, вероятно белкового характера. Антибиотик действует на грам-положительные и грам-отрицательные бактерии и на асцитную опухоль Эрлиха у мышей. Токсичность антибиотика зависит от степени чистоты препарата. У препарата с эффективностью 2000 ед. с./мг при внутривенных впрыскиваниях $LD_{50}$ была 25 мг/кг. По своему антибактерийному спектру и по размерам молекулы антибиотик близок к антиномицетину, от которого отличается однако по своей устойчивости, по способу изоляции и по растворимости в органических растворителях.

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# Study of the Effect of *Bacillus thuringiensis* on Insects

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Some varieties of *Bacillus cereus* are known to be pathogenic for insects. In 1902, Ishiwata in Japan (according to Steinhaus, 1947) isolated *Bacillus sotto* from silk-worms. Berliner (1915) described a spore-forming rod isolated from flour moths *(Ephestia kühniella)* which he named *Bacillus thuringiensis*. Metalnikov et al. (1928) described *B. pyraustae* and *B. pyrenei*, *B. italicum* and *B. cazaubon* (1930) isolated from *Pyrausta nubilalis* and *B. galechiae* (1932), isolated from *Galechia gossypiella*. Toumanoff and Vago (1951) described *B. alesti*, isolated from *Bombyx mori*. It was assumed that all these strains corresponded both morphologically and culturally to the saprophytic *Bacillus cereus* and that they differed from this bacillus only in their pathogenicity for insects, chiefly *Lepidoptera*.

Because of their pathogenic properties, these strains, especially *Bacillus cereus* var. *thuringiensis*, have been investigated in a number of laboratories in Hungary, Yugoslavia, France, the USA and Canada, with reference to their possible utilization as insecticides. The communication of Hannay (1953) drew attention to the formation of paracentral crystalline inclusions by *Bacillus thuringiensis*, which had already been observed in this variety by Berliner in 1915 and by Mattes in 1927. Later, inclusions were also found in the other varieties of *Bacillus cereus* pathogenic for insects (*B. sotto*, *B. alesti* and other, unidentified varieties) and their relationship to pathogenicity for insects was therefore investigated. Hannay (1953), when the composition of the inclusions was still unknown, assumed that they were either of virus origin or that formation of the inclusions was a genetic characteristic of the organism in some way related to the formation of a toxic substance producing septicaemia in insect larvae. Hannay and Fitz-James (1955) found that the inclusions were of a protein nature and that they contained 17 amino acids. Angus (1954) observed the action of inclusions of *B. sotto* dissolved in dilute alkali and came to the conclusion that they were the toxic component of *B. sotto* and when taken orally caused paralysis in silk-worms.

Toumanoff (1951), on the other hand, while attributing a toxic role to crystalline inclusions, regards the enzyme lecithinase as the important toxic factor in these strains. Heimpel (1955) likewise emphasizes the relationship between pathogenicity for insects and the ability to produce lecithinase in various species of the *Bacillus* genus.

The question of the classification of the above-mentioned strains pathogenic for insects is still not altogether clear. *B. thuringiensis* is not described either in the sixth edition of Bergey's Manual or in Krasilnikov's Manual; only Smith et al. (1946) mention *B. thuringiensis*, as a variety of *B. cereus*. According to Steinhaus and Jerrel (1954), *B. thuringiensis* will be included in the seventh edition of Bergey's Manual as an independent species. The other varieties of *B. cereus* differ only slightly and it is quite possible that some are synonymous (e. g. some authors regard *B. sotto* as the Japanese equivalent of *B. thuringiensis*).

The aim of the present work was to investigate the pathogenicity of a Czech strain of *B. thuringiensis* isolated by Dr. Weiser, to ascertain at what stage in its development the insecticidal substance is formed, where it is localised, what its effect is and whether the production of this substance by strains forming inclusions differs from its production by strains in which inclusions are not formed. It was further necessary to elaborate a sensitive method for isolating the inclusions, as the authors who so far studied the question of the pathogenicity of *B. cereus* var. *thuringiensis* (Husz 1927, 1929, 1930, Metalnikov and Chorine 1929a, b, Metalnikov et al. 1931, Steinhaus 1951, Toumanoff 1955, Heimpel 1955) always worked only with sporulated cultures, i. e. with a mixture of spores, inclusions and the remains of vegetative cells, while Angus (1954) dissolved inclusions of *B. sotto* in dilute alkalis; in our experiments this did not prove satisfactory, as it resulted in loss of activity of the filtrate.

## *Materials and Method*

The Czech virulent strain of *B. cereus*, var. *thuringiensis*, was isolated from severely affected larvae of the sweet pepper moth *(Plodia interpunctella)* in 1952. It is characterised by greater virulence than the strains obtained in Hungary and the USA (Weiser and Veber 1954, Žofka 1955). Its diagnostic

characteristics coincide with those of *B. cereus*. Non-virulent strain: When maintained on a meat-peptone agar slant at room temperature for half a year without subcultivation, this strain lost its ability to form inclusions. Both strains were cultured on an agar sporulation medium in Roux bottles at 28° C.

Caterpillars of *Lymantria dispar* and *Euproctis phaeorrhoea* were selected for the experiments; these pests damage deciduous trees, mainly oaks, hornbeams, willows and fruit trees. The caterpillars were reared in large Petri dishes and infected by feeding on willow leaves (in early spring) and later plum leaves smeared with a suspension of infected material. In every experiment, which was carried out in duplicate, twenty caterpillars were used. Some experiments were further repeated using silk-worms *(Bombyx mori)*, which were fed on mulberry leaves.

Isolation of inclusions: This proved very difficult in cultures in which sporulation was complete and which also contained spores and vegetative debris in addition to free inclusions. The specific weight of the inclusions differs only slightly from that of the spores. Differential centrifugation (Behrens and Marti 1955) did not give satisfactory results, nor could it be used even in cases in which the spores in a mixture containing inclusions were allowed to germinate beforehand, in order to increase their substance. As regards time, germination of the spores was very uneven and the part which should have contained inclusions still contained a large quantity of spores which had not yet germinated. Dissolving the inlusions in dilute alkalis, as described by Angus (1954), likewise proved unsatisfactory in our experiments as the inclusions lost their effectiveness on being dissolved. The most satisfactory proved to be the density gradient centrifugation technique (Schneider et al. 1953, Říman 1955). Gradients of 1 ml. of 1.11M, 0.957M, 0.636M and 0.335M saccharose were layered into centrifuge tubes. A culture in which sporulation was complete was washed and suspended in 0.25M saccharose and layered in amounts of 1 ml. into the centrifuge tube over the fourth gradient. Centrifugation was carried out for 25 minutes at 300 G. A ring containing most of the spores and inclusions then fell to the 0.636M gradient and in the upper gradients slightly turbid saccharose remained, which contained almost only inclusions. By removing the upper gradient and washing, a suspension of inclusions containing only very few spores was obtained (50 to 80 : 1).

In their latest communication, Hannay and Fitz-James (1955) describe the isolation of crystalline inclusions by mechanical disruption of the spores and by the germination and autolysis of spore material. This method of isolation was used for making a chemical analysis, however, and not for ascertaining the biological effect of the inclusions.

Isolation of the spores: Three methods were used. A culture in which sporulation was complete was treated for a) 10 minutes at 112° C, at 0.5 atm. overpressure, b) 24 hours with 0.1N NaOH, c) 24 hours with 1% pepsin in a N/20 HCl medium. The protein inclusions were either destroyed or completely dissolved. The germinating capacity of the spores was tested by inoculating on to meat-peptone agar. The same concentration was always maintained in the suspensions, measured by the degree of turbidity.

Determination of antibiotic properties: This was carried out by the plate assay method, using agar blocks (a few pieces of agar of 5 mm. diameter) taken from a fully developed culture of *B. thuringiensis* on nutrient agar.

## *Results*

### Influence of the Age of the Culture on Production of the Insecticidal Substance of *Bacillus thuringiensis*

In these experiments a study was made of the stage at which the insecticidal substance develops.

Table 1 shows that the action of a young, 8-hour-old culture containing only vegetative cells is very weak. The first caterpillars did not die until the fourth day and massive death did not occur until the eighth day. A post mortem examination showed that the cells had proliferated in the weakened organisms sufficiently to cause septicaemia. The most virulent were the cells of a fully developed 7-day-old culture composed of free spores, inclusions and vegetative debris; this caused death in 50% of the caterpillars within three days and in 100% within six days. The results permit the assumption that the effective insecticidal substance develops during the period of sporulation.

### Comparison of the Effect of Spores and Inclusions of the Virulent Strain of *Bacillus thuringiensis*

Since the experiments described above do not show whether the pathogenic agent is the spores or the inclusions which are released at the same time as the spores,

Table 1. Death Rate in the Third Instar of Caterpillars of *Euproctis phaeorrhoea* Fed with Cultures of *B. thuringiensis* of Varying Ages

| Age of culture | 8 hours | 16 hours | 48 hours | 7 days |
|---|---|---|---|---|
| Morphology of culture | Vegetative cells | Vegetative cells with signs of spores and inclusions | Part of spores and inclusions released | Free spores and inclusions, vegetative debris |
| Days | Mortality | | | |
| 1 | 0 | 0 | 0 | 5% |
| 2 | 0 | 0 | 5% | 10% |
| 3 | 0 | 5% | 5% | 50% |
| 4 | 10% | 25% | 35% | 80% |
| 5 | 20% | 65% | 75% | 90% |
| 6 | 25% | 85% | 90% | 100% |
| 7 | 45% | 95% | 100% | |
| 8 | 90% | 95% | | |

Table 2. Death Rate in the Third Instar of Caterpillars of *Euproctis phaeorrhoea* Fed with Isolated Spores and Isolated Inclusions

| Days | Spores | | | Inclusions |
|---|---|---|---|---|
| | Inclusions denatured by heating to 112° C | Inclusions dissolved in 0.1N NaOH | Inclusions dissolved in 1% pepsin | Isolated by by density gradient centrifugation technique |
| | Mortality | | | |
| 1 | 0 | 0 | 0 | 10% |
| 2 | 0 | 0 | 0 | 50% |
| 3 | 0 | 5% | 5% | 70% |
| 4 | 15% | 10% | 5% | 85% |
| 5 | 30% | 20% | 10% | 100% |
| 6 | 45% | 35% | 15% | |
| 7 | 55% | 65% | 25% | |

a further investigation was made to compare the effect of spores only and inclusions only on the death of caterpillars.

Table 2 shows that the isolated spores do not have an immediate toxic effect. The caterpillars do not die for 3—4 days, the cause of death being septicaemia following proliferation of the bacilli which develop from the spores in the intestine. After the ingestion of inclusions, however, some caterpillars died on the very first day. By the second day 50% of the caterpillars were dead and 100% died by the fifth day. The toxic effect of the inclusions was evident only a few hours after ingestion. The caterpillars stopped feeding and their movements decreased. In older, more resistant caterpillars, damage to intestinal function was manifested by the intestine remaining full and by cessation of peristalsis, while in younger, less resistant caterpillars, diarrhoea occurred and the anus became stuck to the underlying surface. Susceptibility of the caterpillars to infection decreased with age. A loss of appetite was observed when the leaves were smeared with a large dose of spores or whole

culture, and the caterpillars fed with infectious material were therefore subjected beforehand to starvation.

Caterpillars infected with spores died from septicaemia. In caterpillars infected with inclusions, normal microflora appeared in the intestine in the first hours after feeding; later, germination of the spores accompanying the inclusions in small quantities occurred (proportion of inclusions to spores 50—80 : 1), while prior to death vegetative forms even penetrated into the haemolymph. Paralysis in the first hours of infection was not therefore accompanied by septicaemia.

Prior to death the caterpillars were soft in consistency and the epidermis acquired a darker tinge. After death the internal organs rapidly disintegrated and the haemolymph dried up.

### Effect of a Strain of *Bacillus thuringiensis* not Forming Inclusions

The above results demonstrated that the inclusions constitute the toxic component of the virulent strain. It was therefore of interest to ascertain the effect of a strain of *B. thuringiensis* which had undergone degeneration and lost the ability to form inclusions. Experiments with this strain showed that when given orally it is non-virulent both in the form of young, vegetative cells (16 hours old) and also in the

Table 3. Death Rate in the Third Instar of Caterpillars of *Euproctis phaeorrhoea* Fed with Inclusions of the Virulent Strain and Spores of the Non-virulent Strain of *B. thuringiensis*

| Days | Inclusions of virulent strain | Inclusions of virulant strain + spores of non-virulent strain | Inclusions of virulent strain + spores of virulent strain | Spores of non-virulent strain |
|---|---|---|---|---|
| | Mortality | | | |
| 1 | 15% | 5% | 5% | 0 |
| 2 | 55% | 15% | 20% | 0 |
| 3 | 80% | 30% | 40% | 0 |
| 4 | 95% | 75% | 85% | 0 |
| 5 | 100% | 90% | 90% | 0 |
| 6 | | 100% | 100% | 5% |

Table 4. Death Rate in the Third Instar of Caterpillars of *Bombyx mori* Fed with Inclusions and Spores of *B. thuringiensis*

| Hours | Inclusions of virulent strain | Inclusions + spores of virulent strain | Inclusions of virulent strain + spores of non-virulent strain | Spores of non-virulent strain | Spores of virulent strain |
|---|---|---|---|---|---|
| | Mortality | | | | |
| 2 | 0 (20% paralysis) | 5% (10% paralysis) | 0 (30% paralysis) | 0 | 0 |
| 19 | 50% (20% paralysis) | 60% (10% paralysis) | 40% (35% paralysis) | 0 | 0 |
| 26 | 100% | 95% | 95% | 0 | 0 |
| 39 | | 100% | 100% | 0 | 0 |

form of spores from a fully developed 7-day-old culture, which pass through the intestine in the excrements with their ability to germinate still intact, without causing septicaemia (table 3, 4).

Infection with vegetative cells of the non-virulent strain did not cause death. These experiments lend support to the view that inclusions of the pathogenic strain merely induce germination of the spores in the intestine of the caterpillar which results in septicaemia.

In experiments in which inclusions were added to the spores of the non-virulent strain, the death rate among infected caterpillars of *Euproctis phaeorrhoea* did not exceed the death rate among the control caterpillars to which only inclusions were administered (table 3, 4).

### Effect of *Bacillus thuringiensis* on Silk-worm (*Bombyx mori*)

Caterpillars in the third instar were very susceptible to infection with *B. thuringiensis*. Within only two hours after ingesting infected leaves they remained motionless on their sides and began to die. A microscopic examination of the haemolymph and contents of the intestine confirmed that no germination of the spores had occurred within this short period and that death was the result of intoxication (table 4).

### Antibiotic Properties of *Bacillus thuringiensis*

In the past few years it has been discovered that some strains of the species *B. cereus* produce antibiotic substances. An attempt was therefore made to ascertain whether the strain of *B. thuringiensis* used in these experiments produced an antibiotic, and if so, whether this was related to its toxic effect on caterpillars.

The plate assay method demonstrated that both strains—the strain forming inclusions and virulent for caterpillars and the non-virulent strain without inclusions—produce an antibiotic which acts on some gram-positive micro-organisms: *B. subtilis* (15 mm. zone), *Sarcina lutea* (18 mm.) and *Staphylococcus aureus* (12 mm.) and not affecting the gram-negative micro-organisms *Serratia marcescens* and *Escherichia coli*. Since the antibiotic was produced in the first hours of growth (6-hour-old culture), before inclusions are formed, and was also produced by the strain not forming inclusions, it may be assumed that the antibiotic is not identical with the inclusions.

## *Discussion*

The present communication, dealing with the problem of ascertaining the effect of spores and inclusions of *B. thuringiensis* on caterpillars demonstrates for the first time the use of a relatively very pure concentrated suspension of inclusions isolated mechanically by a density gradient centrifugation technique. In agreement with views expressed in the literature it was found that the toxic substance does not develop until during the sporulation period. The results given in tab. 2, however, show a basic difference between the effect of isolated inclusions and spores on mortality in caterpillars and show that the inclusions are the main toxic component. The question thus remains of how far other factors (e. g. lecithinase) may participate in the effect of the inclusions.

The observation that the inclusions are the main bearers of toxicity was also confirmed in experiments with a strain of *B. thuringiensis* which had lost the ability to form inclusions. This strain was non-virulent for caterpillars. Toumanoff (1955) also described two strains of *Bacillus sotto* which differed in their virulence for the

silk-worm and observed inclusions only in the virulent strain. By passaging *B. cereus*, var. *alesti* on alkaline agar with a pH of 9.0—9.5, Toumanoff also obtained a culture in which inclusions were absent. This culture was less toxic than the initial culture. On returning the culture to neutral agar, inclusions were again formed in some cases. It may be assumed that in the alkaline medium the inclusions were dissolved and thereby diluted, resulting in a decrease in their toxicity and perhaps a partial degradation of their toxic agent. This observation is in agreement with our own experiments in which the dissolving of inclusions in 0.1 N NaOH always led to the loss of virulence of the filtrate. It differs, however, from the results of Angus (1954), who in the case of *Bacillus sotto* succeeded in dissolving the inclusions in dilute alkali and thus obtained an active, spore-free filtrate which produced paralysis in silk-worms within 2—4 hours.

*Summary*

1. A method was elaborated for isolating inclusions from a mixture of spores, inclusions and vegetative debris by means of a density gradient centrifugation technique. The proportion of inclusions to spores was from 50 to 80 : 1.

2. It was found that the crystalline inclusions formed in the cells of *Bacillus thuringiensis* during sporulation and released from the cells at the same time as the spores, have a strong insecticidal action. They have a toxic effect on caterpillars within a few hours after ingestion (immobilisation of intestinal function, reduced mobility) and cause death in the third instar of caterpillars of *Euproctis phaeorrhoea* within five days and in those of *Bombyx mori* within 24 hours.

3. Spores alone do not have an immediate toxic effect, but cause septicaemia after a few days.

4. A strain of *Bacillus thuringiensis* which has undergone degeneration and has lost the ability to form inclusions is not virulent for caterpillars of *Euproctis phaeorrhoea*, *Lymantria dispar* and *Bombyx mori* when given by mouth. If, however, inclusions from the virulent strain are substituted, infection with the degenerated strain has the same effect as that with the virulent strain.

5. The strain of *Bacillus thuringiensis* investigated produces an antibiotic substance which is not identical with the inclusions.

*(Table XXII)*

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## Изучение действия Bacillus thuringiensis на насекомых

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*Резюме*

Исследовалась спорообразующая бактерия Bacillus thuringiensis, как известно, патогенная для насекомых. В соответствии с данными литературы было установлено, что токсичное для насекомых вещество возникает у B. thuringiensis только в период спорообразования (табл. 1). Клетки этого штамма содержат в этот период, кроме спор, также кристаллические включения (рис. 1). Была разработана методика выделения включений из смеси спор, включений и остатков вегетативных клеток использующая градиентную технику центрифугирования. Найденное нами соотношение между включениями и спорами было 50—80 : 1. Для выделения спор тельца включений денатурировались или нагреванием (до 112 °C), или пепсином, или же 0,1 N-NaOH. Из результатов, приведенных на табл. 2, очевидно, что существует принципиальная разница между токси-

ческим действием на гусениц отдельно включений и отдельно спор и что главной токсической составной частью являются включения. Включения оказывают на гусениц токсическое действие уже через несколько часов после их заглатывания (иммобилизация кишечника, понижение подвижности), вызывают гибель 3-ей стадии гусениц Euproctis phaeorrhoea Don. в течение 5 дней (табл. 2), а 3-ей стадии гусениц Bombyx mori — в течение 24 часов (табл. 4). Изолированные споры не оказывают немедленного токсического действия, но вызывают септикемию через несколько дней.

Наблюдение, что инклюзии являются основным носителем токсичности, было подтверждено также опытами со штаммом B. thuringiensis, который утратил способность образовать тельца включений. При введении per os этот штамм не был вирулентным для гусениц Lymantria dispar, Euproctis phaeorrhoea Don. и Bombyx mori. Если к спорам невирулентного штамма прибавлялись тельца включений вирулентного штамма, то полученная смесь оказывала такое же действие, как и первоначальный вирулентный штамм (табл. 4).

Было установлено, что исследуемый штамм B. thuringiensis образует антибиотическое вещество, которое не тожественно с тельцами включений.

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# Darstellung von mit $^{131}J$ markierten Eiweissstoffen

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Mit $^{131}J$ markierte Eiweissstoffe besitzen in Biologie, Biochemie und Medizin grosse Bedeutung, vor allem für Stoffwechseluntersuchungen. Für die meisten Zwecke ist eine möglichst hohe Radioaktivität des mittels $^{131}J$ markierten Eiweissstoffes bei minimalem Jodgehalt erforderlich, um die ursprünglichen Eigenschaften des Eiweisses zu erhalten. Nach Francis verursacht ein Gehalt von 0,32 bis 0,87 % Jod im Eiweiss keine Veränderung der Antigeneigenschaften (Francis und Mitarb. 1951). Bei der Jodierung ist es notwendig, immer möglichst schonende Bedingungen zu wählen und den Jodüberschuss quantitativ aus der Eiweisslösung zu beseitigen, ohne dass eine Strukturveränderung erfolgt.

Die Markierung von Eiweiss mittels $^{131}J$ beruht auf der Jodierung des Tyrosin- bzw. Histidin-Restes. Der mögliche Jodierungsgrad hängt vom Gehalt dieser zwei Aminosäuren im Eiweissmolekül ab. Menschliches $\gamma$-Globulin enthält 6,7 % Tyrosin und 2,5 % Histidin, menschliches Albumin 4,7 % Tyrosin und 3,55 % Histidin, Pferde-Albumin 4,7 % Tyrosin und 4,02 % Histidin.

Bei den üblichen Methoden der Eiweissjodierung wird Radiojod zusammen mit dem Träger in der entsprechenden Menge von Kaliumjodid (Hughes 1950) nach der Gleichung

$$J_2 + KJ = KJ_3$$

gelöst. Aus dieser Gleichung folgt, dass eine möglichst geringe Trägermenge gewählt werden muss, falls gute Jodierungsausbeuten erzielt werden sollen. Ein Nachteil dieser Methode besteht darin, dass für die Jodierung nur 33 % des Jodes ausgenutzt werden können, wie aus der Gleichung

$$KJ_3 + RH = KJ + HJ + RJ$$

folgt, wobei R den Phenylrest im Tyrosinmolekül bedeutet. Diese Jodierungsmethode benutzte eine Reihe von Autoren unter Anwendung verschiedener Pufferlösungen. In den meisten Fällen wurde eine Gesamtausbeute von 2—12 % bei der Jodierung erreicht.

Francis und Mitarbeiter (1955) befassten sich mit der Methode der Markierung von Eiweissstoffen mittels Jod mit dem Ziel der Ausbeutesteigerung. Es gelang den Autoren die Menge des Kaliumjodid-Trägers herabzusetzen, indem sie die Oxydation des Kaliumjodides mittels Kaliumjodat nach der Gleichung

$$5\,KJ + KJO_3 + 6\,HCl = 6\,KCl + 3\,H_2O + 3\,J_2$$

ausführten. Bei dieser Reaktion ist nur ein kleiner Überschuss an Kaliumjodid erforderlich, um das entstandene Jod in Lösung zu erhalten. Die Autoren erzielten bei der Jodierung von Eiweissstoffen eine Ausbeute von 35 %.

Tabelle 1.

| Nr. | Eiweiss g | NaJ 0,02 M ml | $NaNO_2$ 0,02 M ml | HCl 0,1 N ml | $^{131}J$ ml | mc | NaOH 0,1 N ml | Puffer 0,2 M ml | Jodierungsdauer in Stunden | $H_2O_2$ 30 % ml | Gesamtvolumen ml | Anfang $10^6$ i/Min. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1,0 | 8 | 8 | 6 | 1,5 | 1,50 | 3 | 10 | 20 | — | 50 | 9,15 |
| 2 | 1,0 | 8 | 8 | 6 | 1.5 | 0,06 | 3 | 10 | 4 | 0.3 | 50 | 0,74 |
| 3 | 1.0 | 8 | 8 | 6 | 1.5 | 0.06 | 3 | 20 | 20 | 0.3 | 50 | 0,74 |
| 4 | 1.0 | 8 | 8 | 6 | 1.0 | 0,06 | 3 | 10 | 1 | 0,6 | 50 | 0,66 |
| 5 | 1,0 | 3 | 3 | 6 | 0,2 | 0,06 | 3 | 24 | 24 | 0,2 | 42 | 0,77 |
| 6 | 1,0 | 3 | 3 | 6 | 0,2 | 0,06 | 3 | 10 | 24 | 0,2 | 42 | 0,77 |
| 7 | 1,0 | 3 | 3 | 6 | 0,2 | 0,06 | 3 | 10 | 24 | 0,2 | 42 | 0,72 |
| 8 | 1,0 | 3,3 | 3,3 | 6 | 0,2 | 0,04 | 3 | 10 | 20 | 0,2 | 42 | 0,28 |
| 9 | 1,0 | 3 | 3 | 4 | 0,2 | 0,03 | 3 | 10 | 20 | 0,2 | 42 | 0,21 |
| 10 | 1,0 | 3 | 2 | 4 | 0,2 | 0,03 | 3 | 10 | 20 | 0,2 | 42 | 0,18 |
| 11 | 1,0 | 3 | 3 | 6 | 0,2 | 0,02 | 3 | 10 | 20 | — | 42 | 0,12 |
| 12 | 1,0 | 3 | 3 | 6 | 0.2 | 0,03 | 3 | 10 | 6 | 0,2 | 42 | 0,22 |
| 13 | 1,0 | 3 | 3 | 6 | 0,2 | 0,06 | 3 | 10 | 24 | 0,2 | 42 | 0,76 |
| 14 | 1,0 | 1 | 1 | 2 | 0,2 | 0,06 | 1 | 10 | 24 | 0,2 | 25 | 0,61 |
| 15 | 1,0 | 0,3 | 0,3 | 0,9 | 0,2 | 0,12 | 0,45 | 10 | 50 | 0,2 | 50 | 1,07 |
| 16 | 1,0 | — | 1 | 1 | 0,2 | 0,03 | 0,5 | 10 | 20 | 0,2 | 28 | 0,26 |
| 17 | 0,1 | 0,01 | 0,01 | 0,7 | 1,6 | 3,0 | 0,3 | 1 | 24 | 0,04 | 6,24 | 22,6 |
| 18 | 0,1 | 0,08 | 0,08 | 0,7 | 0,3 | 0,5 | 0,3 | 1 | 24 | 0,04 | 4,94 | 3,88 |
| 19 | 0,3 | 0,25 | 0,25 | 2 | 1,85 | 3,0 | 1 | 3 | 24 | 0,1 | 11,5 | 23,3 |
| 20 | 1,0 | 3 | 3 | 6 | 0,5 | 0,5 | 3 | 10 | 24 | 0,2 | 78 | 1,77 |
| 21 | 1,0 | 3 | 3 | 6 | 0,5 | 0,5 | 3 | 10 | 24 | 0,2 | 78 | 1,77 |
| 22 | 1,0 | 0,51 | 0,51 | 1,53 | 0,85 | 2,25 | 0,77 | 2,2 | 24 | 0,17 | 15,1 | 12,5 |
| 23 | 1,0 | 0,54 | 0,54 | 1,63 | 4,2 | 11,1 | 0,9 | 4 | 24 | 0,18 | 21 | 57,5 |
| 24 | 1,0 | 0,83 | 0,83 | 1,6 | 2,35 | 5,0 | 0,9 | 12,5 | 24 | 0,36 | 37,5 | 39,6 |
| 25 | 1,0 | 0,75 | 0,75 | 2,25 | 3,25 | 10 | 1,13 | 3,25 | 24 | 0,25 | 28 | 79,5 |
| 26 | 0,1 | 0,09 | 0,09 | 0,75 | 4 | 10 | 0,4 | 10 | 24 | 0,2 | 31 | 79 |
| 27 | 0,1 | 0,16 | 0,16 | 1,6 | 20,4 | 16 | 0,8 | 12 | 24 | 0,08 | 40 | 124 |

Versuche 1—19 wurden mit Rinder-$\gamma$-Globulin ausgeführt, Oxydationsdauer 1 Stunde.
Versuche 22—27 wurden 2 Stunden oxydiert.

Eine weitere Herabsetzung der Trägermengen wird durch die Extraktion des Jodes mittels Äther aus dem Oxydationsgemisch ermöglicht (Roche 1951). Die Anwendung von Jod bei der Jodierung erhöht jedoch nicht nur die Gefahr der Jodsublimation beim Abdampfen des Äthers, sondern auch die Möglichkeit der Eiweissdenaturierung.

Nach einem anderen Verfahren kann Jod aus Jodid durch Oxydation mittels Wasserstoffperoxyd in Freiheit gesetzt werden (McFarlane 1956). Die Jodierung verläuft bei neutralem pH 7,5 in 0,9 % NaCl-Lösung mit einer Ausbeute von 24 %. Eine ähnliche Methode benutzte Gilmore (1954), welcher Natriumjodid mittels Persulfat oxydierte und bei Jodierung von Serum-Albumin in Phosphatpuffer bei Anwesenheit von Guanidin eine Ausbeute von 90 % der Theorie (bzw. 45 % der eingesetzten Jodmenge) erhielt. Für die Oxydation kann ferner Natriumnitrit in saurer Lösung verwendet werden (Shulman und Tagnon 1950).

Für die Abtrennung des Eiweiss von unverbrauchtem Jod und Salzen werden Fällungsmethoden, z. B. mittels Essigsäure (Banks u. a. 1951), Dialyse (Melcher und Masouredis 1951) event. auch in Anwesenheit von Ionenaustauschern (Stern-

| Ende $10^6$ i/Min. | Ausbeute % | % J im Eiweiss | Anmerkung |
|---|---|---|---|
| 1,98 | 22 | 0,44 | Rinder-$\gamma$-Globulin, 2 × mit Benzol ausgeschüttelt |
| 0,22 | 29 | 0,58 | dtto |
| 0,39 | 53 | 1,06 | dtto |
| 0,24 | 49 | 0,5 | dtto |
| 0,41 | 52 | 0,36 | dtto |
| 0,39 | 51 | 0,36 | Rinder-$\gamma$-Globulin dialysiert |
| 0,33 | 45 | 0,36 | Rinder-$\gamma$-Globulin 6 × mit Benzol ausgeschüttelt und voroxydiert |
| 0,18 | 67 | 0,5 | Globulin 5 × mit Benzol, 4 × mit $CCl_4$ ausgeschüttelt und voroxydiert |
| 0,13 | 64 | 0,45 | dtto |
| 0,11 | 65 | 0,3 | Globulin 10 Stunden dialysiert |
| 0,04 | 30 | 0,4 | Globulin 4 × mit Benzol ausgeschüttelt |
| 0,07 | 31 | 0,3 | dtto |
| 0,35 | 47 | 0,37 | Globulin 4 × mit Benzol ausgeschüttelt und voroxydiert |
| 0,31 | 50 | 0,12 | dtto |
| 0,46 | 43 | 0,04 | dtto |
| 0,05 | 19 | *) | dtto |
| 11,0 | 49 | 0,1 | Globulin mit Benzol extrahiert |
| 1,74 | 45 | 0,09 | dtto |
| 13,3 | 57 | 0,11 | dtto |
| 0,33 | 19 | 0,15 | Rinderalbumin mit Benzol extrahiert und voroxydiert |
| 0,32 | 18 | 0,15 | Rinderalbumin mit Benzol extrahiert |
| 11,6 | 93 | 0,13 | Pferdealbumin 24 Stunden dialysiert |
| 50,0 | 87 | 0,13 | dtto |
| 13,4 | 20 | 0,06 | Menschliches Albumin, weniger rein, 24 Stunden dialysiert |
| 51,5 | 65 | 0,12 | Menschliches Albumin rein, 24 Stunden dialysiert |
| 3,6 | 4,6 | *) | Tuberkulin 24 Stunden dialysiert |
| 21,0 | 21,0 | *) | BCG 1 24 Stunden dialysiert |

Bei Versuch 20 und 21 betrug die Oxydationsdauer 1 Stunde.

*) Der Jodgehalt im Eiweiss war geringer als 0,01 %.

berg u. a. 1955) benutzt, wodurch der Dialysenverlauf beschleunigt wird. Die Dialysiermethoden arbeiten im allgemeinen schonender.

Da die in der Literatur angeführten Ergebnisse stark schwanken und die Arbeitsmethoden häufig nur ungenügend reproduzierbar sind, befassten wir uns mit der Methodik der Jodierung von Eiweissstoffen eingehender zwecks Ausarbeitung einer verlässlichen Methode, welche hohe Ausbeuten und Erhaltung der ursprünglichen Eigenschaften der Eiweissstoffe ermöglicht.

### *Material und Methodik*

Für die Versuche wurde Rinder-$\gamma$-Globulin, Ei-, Pferde-, Menschen- und Rinder-Albumin und zwei Tuberkulinpräparate benutzt. Für die Jodierung wurden folgende Lösungen verwendet: 0,02 M NaJ; 0,02 M $NaNO_2$; 0,1 N HCl; 0,1 N NaOH; 0,2 M Phosphatpuffer pH 7,5; 20 % $H_2O_2$.

Die 10% Eiweisslösung wurde vor der Jodierung entweder durch wiederholtes Ausschütteln mit 0,5 Vol. Benzol zwecks Beseitigung event. vorhandener phenolischer Stabilisatoren oder durch Dialyse gegen 0,9 % NaCl unter Rühren bei 5° C während 24 Stunden gereinigt. In manchen Fällen erfolgte eine Voroxydation der Eiweisslösung, indem zu dem gereinigten Eiweiss 0,2 ml 30% $H_2O_2$ zugesetzt und 2 Stunden bei 20° stehen gelassen wurde.

Für die Jodierung wurde die in Abb. 1 dargestellte Apparatur benutzt.

In einen 20 ml-Erlenmeyer-Schliffkolben wurde 0,02 M NaJ-, $^{131}J$- und 0,02 M $NaNO_2$-Lösung pipettiert (Tab. 1). Aus dem Scheidetrichter wurde 0,1 N HCl-Lösung zugesetzt. Die Lösung wurde 1 Stunde bei Zimmertemperatur mit elektromagnetischem Rührer gerührt. Nach beendeter Oxydation wurde aus dem Scheidetrichter 0,1 N NaOH-Lösung zwecks Neutralisierung überschüssiger HCl zugesetzt. Hierauf wurde die Eiweisslösung und der Phosphatpuffer in den Scheidetrichter pipettiert, diese Lösung binnen einigen Minuten unter intensivem Rühren zu der Jodierungslösung zulaufen gelassen und 30 % Wasserstoffperoxyd zugesetzt. Die Jodierung verlief dann unter dauerndem Rühren bei Raumtemperatur während einiger Stunden. Nach beendeter Jodierung wurde der Kolbeninhalt in einen Dialysierschlauch überführt und unter Rühren bei 5° C gegen 2 l einer 0,9% NaCl-Lösung dialysiert. Das Dialysat wurde 2 bis 3mal in Intervallen à 24 Stunden ausgetauscht.

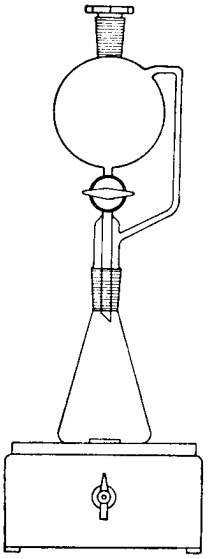

Abb. 1.

Nach beendeter Jodierung wurde das Volumen der Lösung gemessen und in einem aliquoten Teil die Radioaktivität bestimmt und auf das Gesamtvolumen umgerechnet. Auf die gleiche Art wurde der Dialysenverlauf bei jedem Austausch des Dialysates verfolgt. Nach Beendung der Dialyse wurde das Volumen erneut bestimmt und das Ergebnis der Endanalyse auf eine event. Volumenänderung korrigiert.

Die markierten Eiweissstoffe wurden der Elektrophorese unterworfen. Die Elektrophorese erfolgte in einer feuchten Kammer an gespannten Filterpapierstreifen mit Veronal-Citrat-Puffer von pH 8,6 bei 250 V während 8 Stunden. Der Nachweis wurde durch Entwickeln des Elektrophoregrammes mittels Bromphenolblau in alkoholischer mit Quecksilber-2-chlorid gesättigter Lösung ausgeführt.

Die Radioaktivität wurde mittels Geiger-Müller-Zählrohr mit Glimmer-Endfenster bestimmt. Die Proben wurden unter gleichen geometrischen Bedingungen gemessen. Die Ergebnisse wurden auf den Hintergrund und Zerfall des Radioisotops korrigiert; Korrektion auf Eigenabsorption musste nicht erfolgen, da die Masse der Proben zu vernachlässigen war.

Die Proben für die Messung wurden bereitet, indem wir 0,1 ml (nach geeigneter Verdünnung) auf ein Aluminiumschälchen von 2 $cm^2$ Fläche pipettierten, 0,1 ml 0,1 N Natriumthiosulfatlösung zusetzten und unter der Infrarotlampe trockneten.

### *Ergebnisse*

Die Ergebnisse der Versuche, in welchen die zugesetzte Trägermenge, die Menge an Radiojod, die Jodierungsdauer und die Menge zugesetzten Wasserstoffperoxyds verändert wurde, sind in Tab. 1 zusammengefasst. Aus der Tabelle folgt der Einfluss der Reinheit des Eiweiss auf die Jodierungsausbeute, was am besten aus Versuch Nr. 24 und 25 hervorgeht, in welchen zwei Albumine von verschiedener Reinheit benutzt wurden. Die besten Ergebnisse werden bei der Jodierung von durch Dialyse gereinigten Eiweissstoffen erzielt. Die Trägermenge beeinflusst die Ausbeute in weiten Grenzen nicht. Nur bei der Jodierung mit dem ursprünglichen Radiojodpräparat, welches ohne Trägerzusatz oxydiert wurde, sind die Ausbeuten besonders niedrig. Die Oxydation von Radiojod und zugesetztem Träger erfordert für den quantitativen Verlauf mindestens 1 Stunde. Als optimale Dauer der eigentlichen Jodierung erwiesen sich 20 bis 24 Stunden. Da bei der Jodierung eine dem an Eiweiss gebundenen Jod äquivalente Jodmenge zu Jodid reduziert wird, ist es möglich, die Ausbeuten durch dauernde Reoxydation des Jodids zu Jod zu erhöhen, was durch Zusatz einer kleinen Menge Wasserstoffperoxyd zu dem Jodierungsgemisch ausführbar ist. Es gelang so, die Ausbeuten bei der Jodierung bis auf 90 % der eingesetzten Menge an $^{131}J$ zu erhöhen. Während des gesamten Versuches erfolgte keine Veränderung des pH, welches sich dauernd auf einem Wert um 7,5 hielt. Für die Reinigung der markierten Eiweissstoffe von überschüssigem Jod eignet sich am besten die Reinigung mittels meist 2 bis 3mal wiederholter Dialyse, wodurch mehr als 99 % des nicht gebundenen Jodes entfernt werden kann. Im Verlauf der

Versuche erfolgte niemals Denaturierung des Eiweiss, wovon wir uns einerseits durch Elektrophorese der Eiweissstoffe, andererseits durch immunologischen Test und Exkretionskurve überzeugten.*)

*Diskussion*

Die im Grossteil der Literatur beschriebenen, relativ niedrigen Jodierungsausbeuten und deren beträchtliche Schwankungen besitzen mehrere Gründe. Dies ist in erster Linie die Reinheit der Eiweissstoffe. Die für die Jodierung bestimmten Eiweissstoffe müssen vollkommen rein sein und dürfen vor allem keine durch Jod oxydierbaren Substanzen enthalten. Meist werden nach verschiedenen Verfahren gefällte Eiweissstoffe benutzt, welche jedoch noch häufig Spuren von Verunreinigungen, vor allem Stabilisatoren, enthalten. So enthielt z. B. ein von uns benutztes $\gamma$-Globulinpräparat 0,3 % Trikresol. Für die Erzielung hoher Ausbeuten ist es vorteilhaft, den Eiweissstoff vor der Jodierung einer Voroxydation mittels einer kleinen Menge Wasserstoffperoxyd während 2 Stunden zu unterwerfen, wodurch mittels Jod oxydierbare Substanzen oxydiert werden, welche bei der Jodierung die für die eigentliche Jodierungsreaktion zur Verfügung stehende Jodmenge herabsetzen würden. Vorteilhafter ist es, die Eiweissstoffe durch Dialyse zu reinigen; die Voroxydation entfällt dann. Ein weiterer wichtiger Faktor ist die quantitative Oxydation des radioaktiven Jods und des zugesetzten Trägers, was durch Wahl einer geeigneten Reaktionsdauer erreicht wird. Die meisten Autoren vermischten die Eiweisslösung im Puffer mit der frischbereiteten Jodierungslösung sofort; nach unseren Erfahrungen erfordert die Oxydation von Jodid zu Jod mindestens 1 Stunde. Bei den für unsere Versuche benutzten Jodkonzentrationen bleibt das Jod nach der Oxydation des Jodids in Lösung, und es ist daher nicht notwendig, mit einem Lösungsmittel oder grösserer Trägermenge zu arbeiten. Es ist unter diesen Versuchsbedingungen nicht ausgeschlossen, dass es zu oxydativen Veränderungen der Eiweissstoffe kommt, diese beeinflussen jedoch nicht deren Verwendbarkeit für immunologische Zwecke.

Für die Erreichung hoher Ausbeuten eignet sich der Zusatz einer kleinen Wasserstoffperoxydmenge zu der Jodierungslösung, welcher das bei der Jodierung entstehende Jodid erneut zu Jod oxydiert. Auf diese Art ist eine beinahe quantitative Ausnutzung des gesamten Jods für die Markierung von Eiweiss erreichbar. Für die Abtrennung von Salzen aus der Lösung der jodierten Eiweissstoffe benutzen die meisten Autoren Fällungsmethoden. Diese Methoden sind mit Verlusten verbunden und gestatten es nicht, mehr als 99 % des ungebundenen Jods zu beseitigen. Es gelang uns in unseren Versuchen auch durch 5mal wiederholtes Fällen nicht, diese Werte zu erreichen. Wir führten die Reinigung daher durch Dialyse gegen 0,9 % NaCl-Lösung bei 5° C aus. Die Dialyse wurde für gewöhnlich 2 bis 3mal à 24 Stunden wiederholt, bis der Jodgehalt in der Eiweisslösung während 24 Stunden um nicht mehr als 0,5 % sank. Auf diese Art gelang es, mehr als 99 % des ungebundenen Jods zu beseitigen.

*Zusammenfassung*

Es wurde eine Methode der Jodierung von Eiweissstoffen ausgearbeitet, welche die einfache Markierung mit $^{131}J$ gestattet. Die Ergebnisse sind stabil und gut reproduzierbar.

*) Wir danken Herrn Dr. Říha aus dem Biologischen Institut für die freundliche Ausführung der immunologischen Teste und der Exkretionskurven.

Die hohen Jodierungsausbeuten sind durch die Reinheit der zur Jodierung benutzten Eiweissstoffe, den quantitativen Verlauf der Oxydation des Jodids zu Jod und den Zusatz einer kleinen Wasserstoffperoxydmenge bei der Jodierung bedingt.

Durch Einhalten der angeführten Bedingungen können Ausbeuten bis zu 90 % der eingesetzten Gesamtmenge an Radiojod erzielt werden.

Für die Beseitigung überschüssiger Salze eignet sich die Reinigung durch Dialyse während 72 Stunden am besten.

Die Anwendung minimaler Trägermengen bei Einhaltung hoher Ausbeuten gestattet die Darstellung von Eiweissstoffen mit einem derartigen Jodgehalt, dass keine Veränderungen ihrer Antigeneigenschaften erfolgen.

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# Приготовление белков меченых $^{131}J$

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*Резюме*

Был разработан метод мечения белков радиоактивным иодом $J^{131}$. Так возможно производить иодацию белков с использованием вплоть до 90% от взятого в опыт количества радиоактивного иода. Получаемые результаты оказываются постоянными и воспроизводимыми.

Для получения высокого % иодации необходимо применять абсолютно чистые белки без примесей и стабилизаторов. Поэтому перед иодацией предварительно производился диализ белков с 0,9% NaCl.

Для полного использования всего иода из раствора для иодации белков необходимо окисление всего иодида без остатка до иода под действием $NaNO_2$ в кислой среде в течение 1 часа.

В связи с тем, что при иодации белков происходит восстановление $J_2$ в количестве, соответствующем связанному иоду, выход иод возможно повысить путем прибавления небольшого количества $H_2O_2$, в результате чего иодид непрерывно переходит в $J_2$, способный к дальнейшей иодации. Так можно добиться полного использования иода. Поэтому бывает возможно работать с очень незначительной концентрацией иода, остающегося в растворе, и нет необходимости в растворителях или в больших количествах субстрата. При лабораторной температуре иодация протекает за 24 часа. После окончания иодации избыточные соли удаляются путем диализа с 0,9% NaCl. Сменяя диализаты трижды в сутки, можно отделить более 99% не вступившего в реакцию иода.

По этому методу была успешно произведена иодация различных сортов глобулина, альбумина и туберкулина. Результаты реакции зависят от содержания тирозина и гистидина. Поэтому у первых 2 белков результаты получаются лучше, чем у туберкулина. Применение минимальных количеств носителя с максимальными результатами позволяет приготовить белки с содержанием иода, которое не оказывает влияния на их антигенные свойства.

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# The Physiology of the Formation of Anthocyanin in Carrot Root Cultures *(Daucus carota* L.*)*

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As long ago as 1941, Gautheret observed that in carrot root cultures cultivated in vitro, anthocyanin was formed in the "pseudothalles" (Gautheret), "Wundhaare" (Magnus), "hojivé vlásky" (Petrů). The cytology of pseudothalles has been described by Petrů (1954). In the further cultivation of these cultures, this characteristic feature, i. e. the ability of individual varieties to form anthocyanin, was used for differentiating individual varieties of carrots. In some varieties, production of anthocyanin in the "pseudothalles" is typical under certain conditions, whereas in others only chlorophyll is produced. Some varieties produce both. This phenomenon is markedly specific in cultures in vitro.

In the present work, the physiology of the formation of these pigments was dealt with and a study made of the relationship between carbohydrate metabolism and the formation of anthocyanin or chlorophyll in various varieties cultured in vitro under the same conditions.

## *Methods*

All the cultures were maintained on 5% saccharose at a temperature of 20° C during the daytime (17° C at night), in the daylight. Samples were always collected for analysis from the same part of the same carrot root, ensuring homogenity of the material used in the experiment. The values given in the tables are the averages from several parallel analyses. Because of the small amounts involved (0.05 — 1 g.), the well-known chromatographic method of the constant addition of samples of individual carbohydrates to the mixture under analysis (Cain 1951, Berry and Cain 1951) was selected for the quantitative determination of carbohydrates. The fresh specimens were carefully weighed and homogenised, adding $CaCO_3$ extracted with hot water and after filtering, cleared with lead acetate. The solution was then filtered and the excess lead ions precipitated with sodium sulphate. The resultant carbohydrate solution was condensed and made up to an exact volume (0.5 — 5 ml.) according to weight, so that the ultimate concentration of carbohydrates was 1 — 5%. From this, 20 $\mu$l. of the extract was applied to Whattman chromatographic paper No. 1. The presence of inorganic salts did not, in general, affect the results, because of their extremely low concentration.

The quantitative evaluation of carbohydrates was carried out by constantly adding a standard solution of 0.1 — 5% glucose, saccharose and fructose. A mixture of n-butanol-acetic acid-water (4 : 1 : 5) was used for developing; sugars were detected by means of an aniline-phthalate mixture. The intensity of the spots was compared visually and the quantitative composition determined on the basis of their area, always taking the average of a number of specimens. The results were calculated and expressed as a percentage of the known amount of fresh weight.

## *Results and Discussion*

It was found that in the varieties tested by us (the edible *Daucus carota*, Selekty polodlouhá and Stupická bílá, used for fodder) a marked decrease occurred in the saccharose content on culturing under the conditions described above (from 2 — 6%

Table 1. Variety: Stupická bílá (fodder).

| Date of taking specimen | % | | |
|---|---|---|---|
| | glucose | saccharose | |
| 11. II. 55 | 1.3 | 3.7 | Start of experiment |
| 16. II. 55 | 1.4 | 1.1 | |
| 22. III. 55 | 1.8 | 0.6 | Formation of chlorophyll |
| 22. III. 55 phloem | 2.9 | 1.0 | |
| 22. III. 55 wood | 0.8 | 0.3 | |

Table 2. Variety: Selekty polodlouhá (edible).

| Date of taking specimen | % | | |
|---|---|---|---|
| | glucose | saccharose | |
| 4. III. 55 | 1.3 | 5.7 | Start of experiment |
| 10. III. 55 | 1.4 | 5.1 | |
| 22. III. 55 | 1.5 | 4.8 | Formation of anthocyanin |
| 22. III. 55 phloem | 1.6 | 3.2 | |
| 22. III. 55 wood | 1.3 | 6.3 | |

to 0.6—4.8%; see tab. 1 and 2). This decrease is manifested particularly markedly in the phloem (from 5.7% to 3.2% or from 3.7% to 1.0%). In varieties forming anthocyanin, an increase in the formation of saccharose was observed in the xylem, as compared with varieties forming chlorophyll, in which a marked decrease in saccharose was apparent in the xylem. Glucose accumulated in the phloem. In contrast to varieties which form mainly anthocyanin, saccharose accumulates in the callus in chlorophyll-forming varieties (1.0% in callus, as compared with 0.3% in the xylem); this is probably due to photosynthetic activity.

In anthocyanin-forming varieties, the proportion of glucose to saccharose changes in the course of production of anthocyanin, which is formed chiefly in "pseudothalles" in the phloem. In the phloem, this change is markedly in favour of glucose (from $^1/_4$ to $^1/_2$), while in the xylem it is in favour of saccharose (from $^1/_4$ to $^1/_5$). This shows that respiratory activity is more intensive in the phloem, saccharose being consumed as a physiologically active sugar, while glucose, as a banal product of metabolism, accumulates. The accumulation of glucose and saccharose in callus tissue in chlorophyll-forming varieties can be explained on the basis of photosynthetic activity. In other yellow and white varieties of fodder carrots studied (Loberišská, Táborská žlutá, Osevy oranžová) a marked decrease in saccharose was observed in all parts of the carrot, while the glucose content rose slightly, or was maintained at the same level.

Marked formation of chlorophyll was observed in cultures of these varieties in vitro. In anthocyanin-forming varieties, far less glucose accumulates in the phloem (from 1.3% to 1.6%), although the decrease in saccharose is approximately the same as in chlorophyll-forming varieties (2.5% and 2.7%). This indicates that some of the glucose must have been used and gives rise to the question of whether glucose may not serve as a substrate for the metabolism of anthocyanin, while in the process of the formation of chlorophyll it is not utilized. There are various opinions on the biogenesis of anthocyanin, but the problem of aromatisation in plants is still open. On the basis of their experiments, the authors incline to the view of the aromatisation of glucose (metabolism of sikimic acid or inositol?). In general it may be said that

the conditions of carbohydrate metabolism in cultures in vitro differ fundamentally in different varieties of carrots and are to a certain extent associated with the possibility of intensive formation of anthocyanin or chlorophyll. Examples of two typical varieties are given in tabs. 1 and 2.

*Summary*

Physiological studies of the formation of anthocyanin in carrot cultures in vitro show that the formation of anthocyanin is a typical property of some varieties and is directly associated with questions of heredity. A study of carbohydrate metabolism shows that tissue which produces anthocyanin displays marked respiratory activity and hoards glucose as a banal sugar, but that much of the latter is probably consumed in the formation of anthocyanin.

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## Физиология образования антоцианов в эксплантатах корня моркови (Daucus carota L.)

И. ХРАСТИЛ и Э. ПЕТРУ

*Резюме*

Изучение физиологии образования антоцианов в моркови, выращиваемой in vitro, показало, что образование антоцианов — это особенность, типичная для некоторых сортов и прямо связанная с вопросами наследственности. Исследования метаболизма глицидов показали, что в ткани, образующей антоциан, наблюдается более интенсивное дыхание и накопляется как обыкновенный сахар глюкоза, большая часть которой однако расходуется, вероятно, для образования антоцианов.

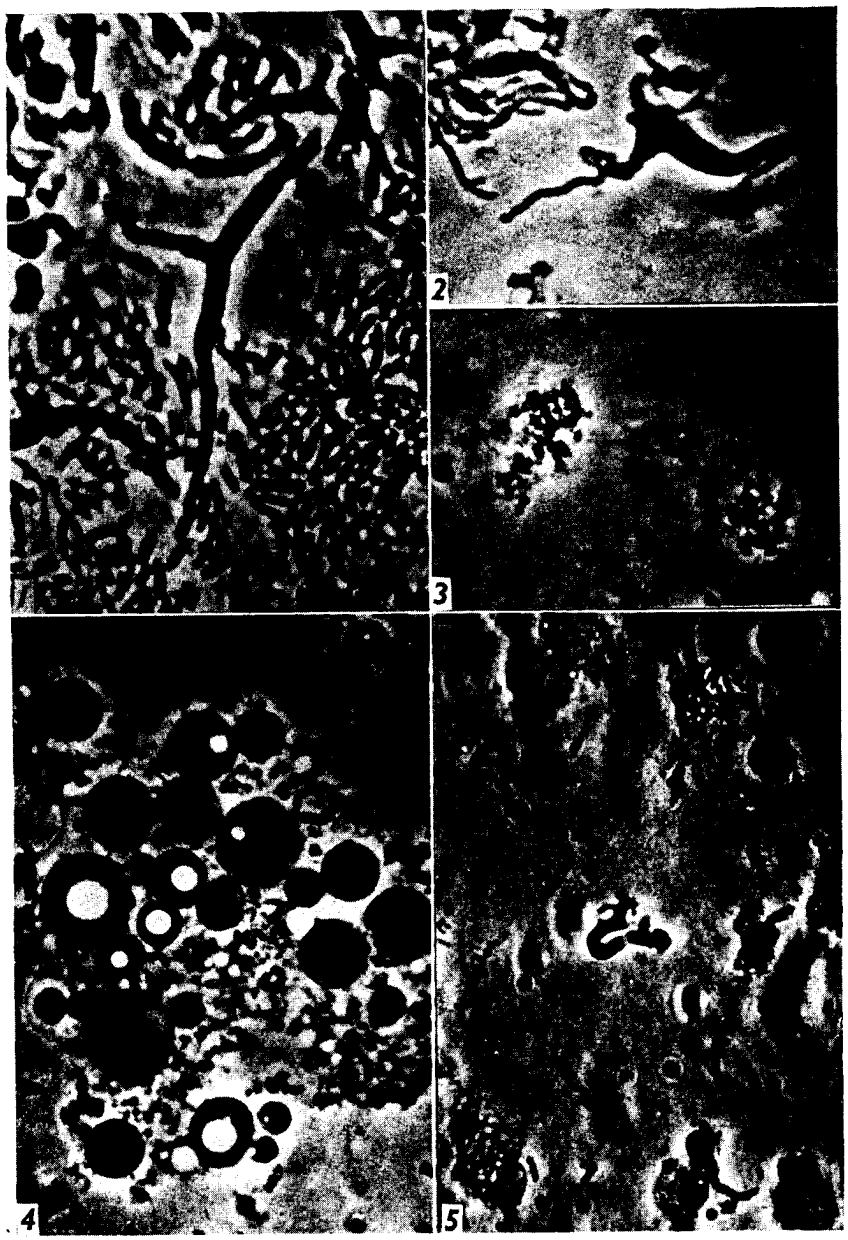



Рис. 1. Разветвленное крупное продолговатое тельце Proteus, с одного конца которого отшнуровываются палочки. Склонность к разветвлению заметна и у остальных КПТ. 2000 ед/мл, 4° С, через 10 дней после посева. Увеличение 1200×.

Рис. 2. Начало отделения палочек от КПТ. Увеличение 1200×.

Рис. 3. Скопления зернистости после распада ККТ. 10.000 ед/мл, 37° С. Увеличение 1500×.

Рис. 4. ККТ в различных стадиях развития через 2¼ часа после посева. 20.000 ед/мл, 22° С. Увеличение 1500×.

Рис. 5. 6000 ед/мл, 4° С, через 10 дней после посева. Некоторые шары еще в состоянии распада, другие образуют КПТ. Увеличение 1000×.

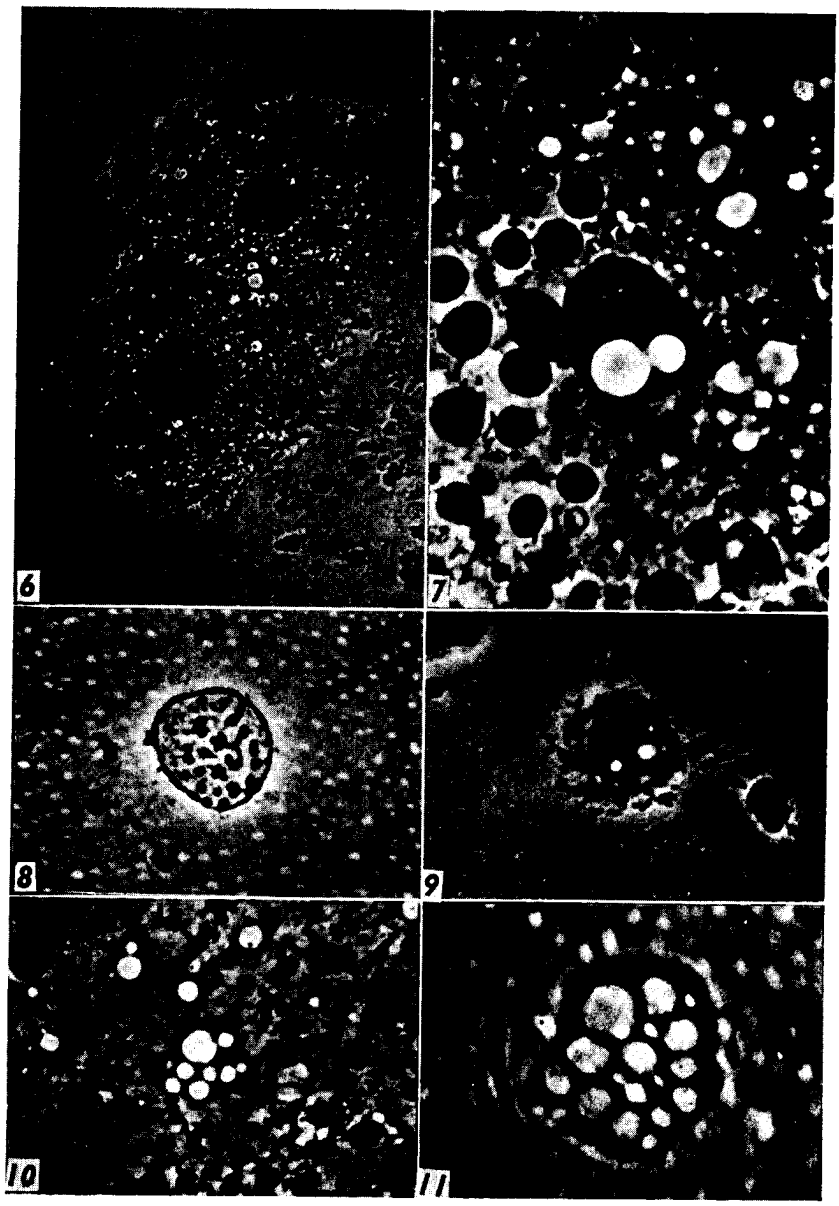



Рис. 6. L-колония Proteus mirabilis. Периферия колонии состоит из регенерировавших палочек. Увеличение 1000×.
Рис. 7. Деталь периферии L-колонии. Пояснения в тексте. Увеличение 1500×.
Рис. 8. Гранулированное ККТ через 24 часа после посева. 40.000 ед/мл, 37° С. Увеличение 1200×.
Рис. 9. Замедленный распад ККТ при 4° С, через 10 дней после посева. Мелкая зернистость на периферии. 6000 ед/мл. Увеличение 1200×.
Рис. 10. Вакуолизация мелких шаров и крупинок через 30 часов после посева. 10.000 ед/мл, 37 °С. Увеличение 1100×.
Рис. 11. Вакуолизированное ККТ Proteus. Увеличение 2000×.

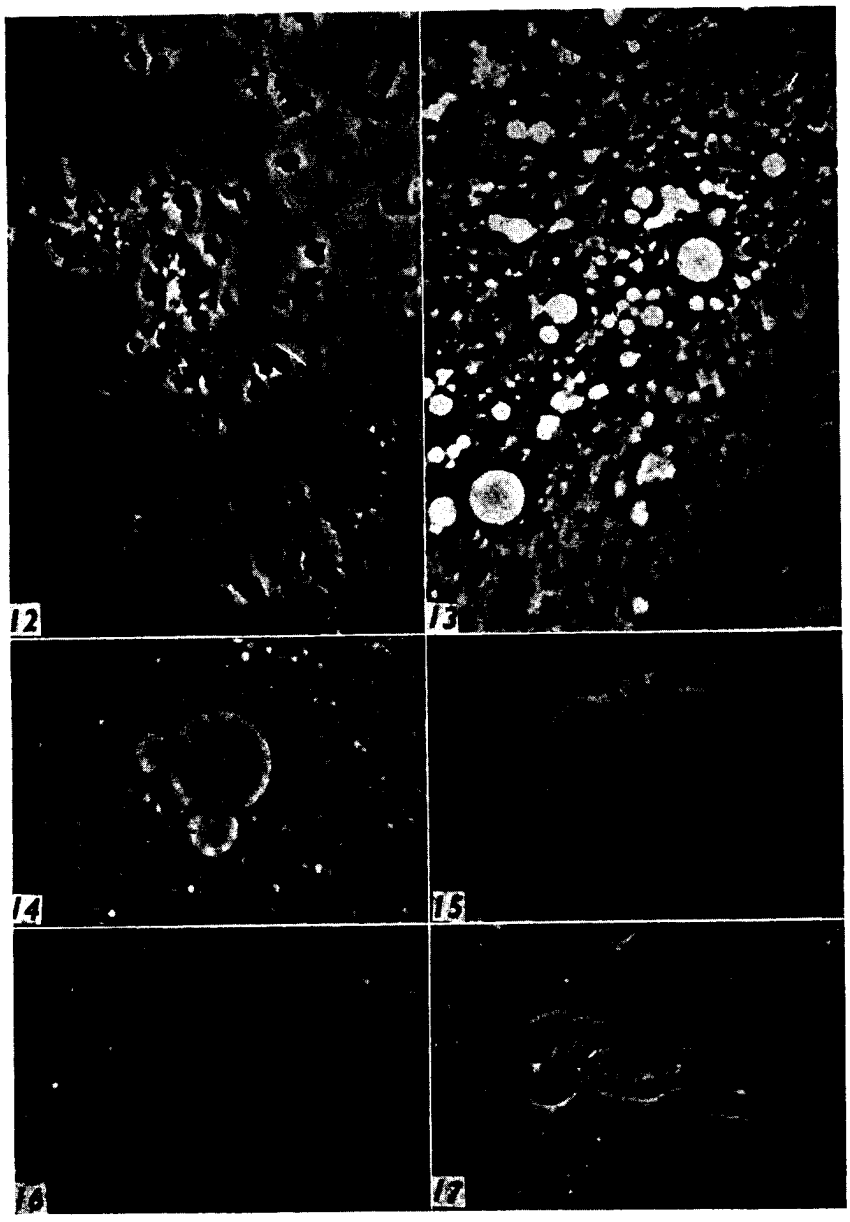


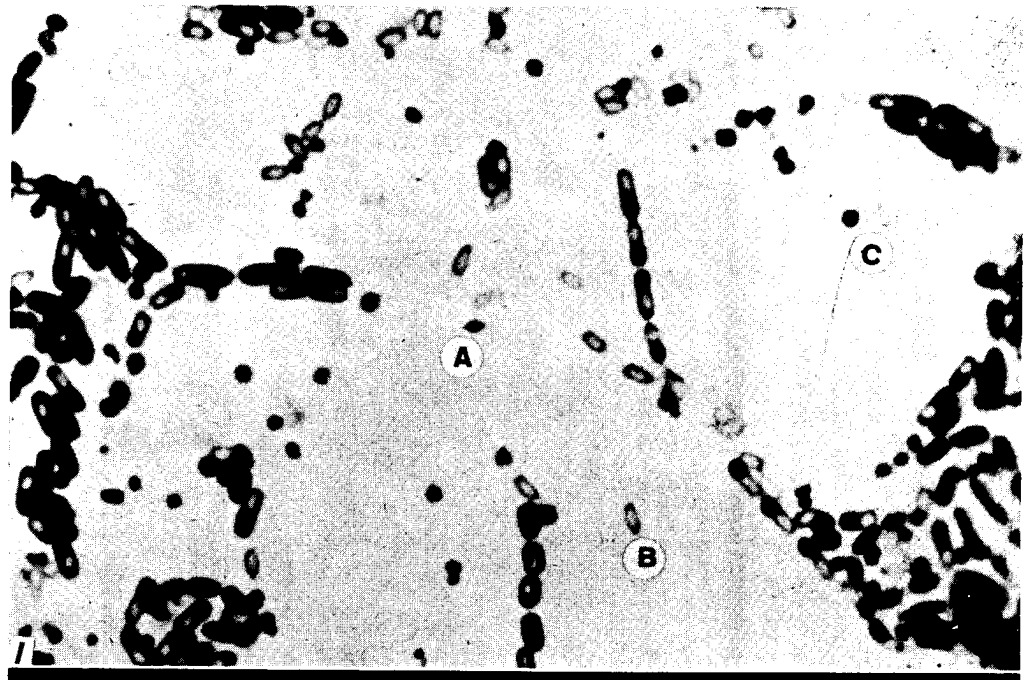

Рис. 12. Зернистость и мелкие шары после распада ККТ через 36 час. после посева. 20.000 ед/мл, 22° С. Увеличение 950 ×.

Рис. 13. Многочисленные вакуоли мелких шаров и зернистости. Увеличение 1100 ×.

Рис. 14, 15. Крупные вакуоли в грануляции после распада ККТ, через 24 дня после посева. 20.000 ед/мл, 22° С. Увеличение 1500 ×.

Рис. 16. Вакуолизированные элементарные тельца L-организмов. Увеличение 1300 ×.

Рис. 17. ККТ и КПТ, возникшие из зернистости и мелких шариков из L-колонии. Увеличение 750 ×.



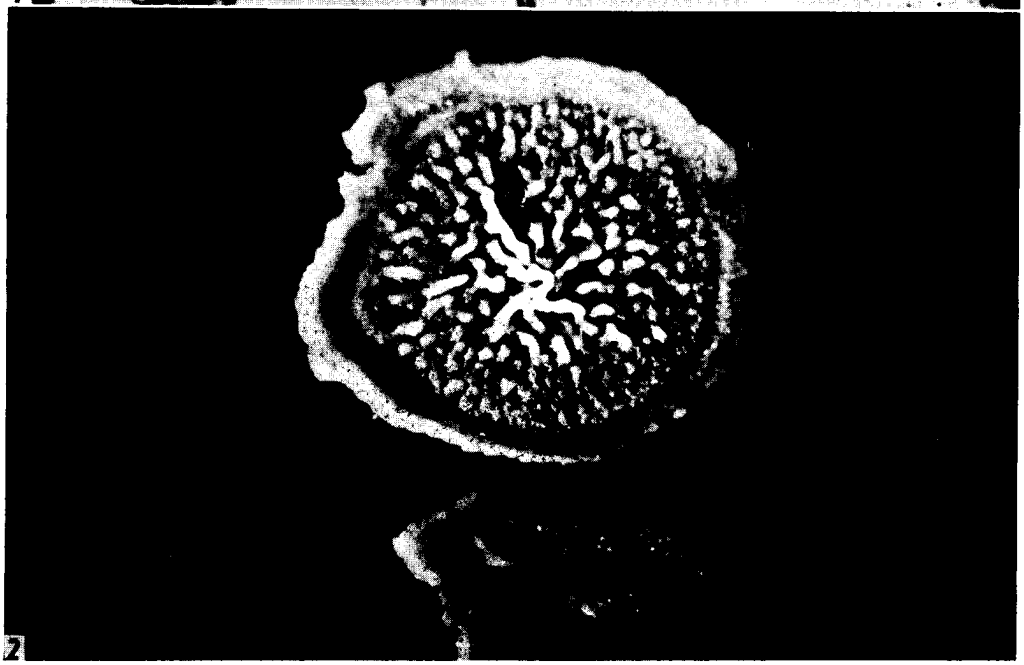




Fig. 1. 72-hour-old culture of virulent strain of *B. thuringiensis* in stage of formation of spores and inclusions. A: typical rhomboid inclusion, B: non-staining spore, C: spore and inclusion before separation. Stained with carbol-gentian violet. Magnification: 2,100×. Photograph by J. Kubec.

Fig. 2. 7-day-old colony of *B. thuringiensis* (MPA, 25° C), with typical appearance following complete sporulation and release of inclusions. Magnification: 7×. Photograph by O. Lysenko.

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